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RADIOACTIVITY AND LUNG CANCER; A CRITICAL REVIEW OF LUNG CANCER IN THE MINERS OF SCHNEEBERG AND JOACHIMSTHAL

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There is a growing conviction in the United States and abroad that radiations emitted by radon inhaled for long periods of time or emitted from radioactive deposits in the lungs will cause cancer of the lung in man. This conviction is based on the fact that primary carcinoma of the lung accounts for 50 percent of the causes of death of the miners of Schneeberg and Joachimsthal. These mines contain radon, particularly those of Joachimsthal, where pitchblende is mined. With the use of ever-increasing amounts of radium in industries such as luminous-paint manufacture and laboratories working with artificially radioactive substances, it is important to know whether radiations from radioactive substances will induce pulmonary tumors. What, then, is the evidence of the carcinogenic properties of radiations with respect to lung tissue? Is there sufficient evidence to warrant the statement that cancer of the lung can be induced in man by radioactive substances? To answer these questions, it is necessary to review and to evaluate the literature on pulmonary cancer in the miners of Schneeberg and Joachimsthal since the latter part of the last century, the literature on the radium and radon content of the mines, and the experimental work carried out on this problem.

LUNG CANCER IN THE MINERS

Schneeberg (in Germany) and Joachimsthal (in Czechoslovakia) are situated in the Erzgebirge, or Ore Mountains, which form the border between Saxony and Bohemia. The mountains are famous for their mineral wealth. Mining started in Schneeberg at the beginning of the fifteenth century (*1*) and in Joachimsthal at the beginning of the sixteenth century (*2*). Besides copper and iron, at first silver was mined, and later cobalt, arsenic, bismuth, and nickel. In the latter part of the last century pitchblende was mined, especially in Joachimsthal, and was used in the manufacture of uranium dyes (*1, 2*). In 1909 the manufacture of radium was started in Joachimsthal, and from 1909 to 1925, 26 gm. of radium element was produced (*3*).

While the mines in Schneeberg were still prosperous in the last century, a more or less rapid decline set in at the turn of the century, which was reflected in the number of active miners. In 1879, 700 to 800 men were employed (*4*); in 1921 the number was reduced to 149, in 1923 to 54 (*1*), and in 1939 was increased to 70 (*5*). No detailed data are available on the number of men working in Joachimsthal. In 1924 there were about 400 miners, and 60 workers in the radium factory (*6*), and in 1939 approximately the same number (*7*).

Many of the miners of these districts are known to die in middle age of a pulmonary disease which the miners call "Bergkrankheit," or mountain disease. As early as the beginning of the sixteenth century, descriptions of the disease can be found in old chronicles. Clinically, the disease manifests itself

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in a persistent cough with viscous discharge, pain in the chest and back, and dyspnea often lasting many years (7 to 20). It occurs in miners that have been working in the mines for over 10 years (*1*, *4*). The "Bergkrankheit" was first diagnosed as cancer of the lung by Härting and Hesse (*4*).

Living, working, and social conditions are bad in the mining districts. Detailed data are available on Schneeberg (*1*, *4*, *6*, *9*). Few are available for Joachimsthal, but it can be assumed that they are of equal order. With recognition of the disease as lung cancer, improvements in these conditions have doubtless been made in recent year (*10*), but their extent is not fully known. The majority of the miners live far away from the mines and have to walk as much as several hours across the windy high plateau to the mines. In many of the Schneeberg mines they have to descend ladders many hundred feet to their working places. The temperature in the mines is between 9° and 16° C., the mines are damp, and during the melting of the snow they are wet. Ventilation is usually sufficient except in blind shafts; it is provided by a gallery connecting all mines (*1*). After work the miners have to climb the ladders again and usually walk home without changing their clothing, which is soaked with sweat. It is no wonder, therefore, that chronic respiratory diseases are common. Ziel (*12*) states that the Joachimsthal miners have to support for 3 hours daily heavy boring hammers on hip or shoulder and that the body is subject to violent concussions.

Härting and Hesse (*4*) were the first to carry out exhaustive and excellently planned investigations of the Bergkrankheit. They reported their findings in 1879. Twenty necropsies were performed. In most of them lymphosarcoma was found at the root of the lung and in one a carcinoma of the bronchial lymph nodes. The lungs were often black with pigmentation. Not rarely multiple cancer nodules were found in the pleura. The bronchial lymph nodes were often enlarged, melanotic, and hard and contained material that looked like coal. Härting and Hesse stated that in 75 percent of all deaths the cause was lung cancer and that the incidence was greater in miners than in masons or carpenters working in the mines. The onset of the disease occurred after about 20 years' work in the mines. Härting and Hesse investigated the dust in the mines and determined that a miner inhaled approximately 6 gm. of dust in 7 hours. They assumed as the cause that inhaled arsenic produced irritation of the lymph nodes in which it collected and that respiratory diseases especially and the bad state of nutrition were predisposing factors in the development of the malignancies. Information on cancer of the lung among the miners of Joachimsthal was not elicited by questioning at that time.

Not until 1913 were new investigations carried out. At this time Arnstein (*13*) reported statistical data showing that from 1875 to 1912, 276 miners had died of cancer of the lung, 64 of tuberculosis, 119 of other lung diseases, and 206 from other causes. Thus the lung-cancer mortality was approximately 40 percent. Arnstein stated that there were probably cancers in those that died of other pulmonary diseases. He (*14*) was the first to diagnose the cancer as squamous-cell carcinoma.

Again a long period elapsed before new investigations were carried out, although Uhlig (*15*) in 1921 and Beyreuther (*16*) in 1924 described isolated cases of lung cancer in the Schneeberg miners and gave histologic findings. Beginning in 1921 Rostoski and Saupe (*1*) carried out an exhaustive clinical investigation while Schmorl (*1*) obtained pathologic material from necropsies. Their investigations were made on 116 miners, 32 invalids, and 6 former miners, 167 factory workers who processed the mined ore (cobalt-dye factory), and 186 people of the surrounding country (not miners). A complete physical examination with radiographs was

made of each person. The average working time of the miners was 25 years, the longest 45 years, and the shortest, 10 years. The clinical picture frequently showed the persistent cough with viscous exudate and the other symptoms mentioned on page 2. Physical examination revealed the presence of consolidation in the upper lung in some cases. The blood picture was more or less normal, with slightly lowered hemoglobin. X-ray examination showed tuberculosis in a few persons, pneumoconiosis in 17 (of whom 4 later developed tumors), and cancer of the lung with pneumoconiosis in 17 others. Those not employed in the mines did not show any indication of tumor. Of the 154 miners, invalids, or former miners, 21 died within 3¼ years. Thirteen of them were necropsied, and the clinical diagnosis of lung cancer was verified. Two of them had left the mines 15 and 22 years before, after working in the mines 10 and 17 years, respectively. With the omission of these 2, 50 percent of the miners who died during this period died of lung cancer. The average age at which cancer was found was 55 years, the oldest man being 69, the youngest 37. In the general population of the mining district, only 2 cases of cancer were found. Histologically, all persons with lung cancer showed epithelial neoplasm: no lymphosarcomas were found. The authors state that the diagnoses of Härting and Hesse (*4*) in 1879 were probably due to misinterpretation. In 9 persons the carcinoma started from large bronchi, in 6 from alveolar tissue, and in the remaining 6 the point of origin could not be determined with certainty. Twelve showed squamous-cell carcinoma, six carcinoma simplex, and three small-cell carcinoma. All showed pneumoconiosis at the site of the tumor, and the authors considered this to be a precancerous lesion. They stated that in Dresden, where anthracosis is frequent, cancer of the lung is also frequent.

Saupe (*17*) in 1933 stated that in the large cities of Saxony, which are very dusty because of the limestone and soft-coal industries, pneumoconiosis and cancer of the lung are frequent. Of all cancer deaths in the city of Dresden from 1923 to 1930, 19.8 percent resulted from cancer of the lung (male 28 percent, female 6.8 percent). However, he (*1*, *17*) considered pneumoconiosis as only one of the factors causing pulmonary tumor. In the case of the Schneeberg miners, he considered also arsenic, previous numerous affections of the respiratory tract, unfavorable living and working conditions, and finally radon and radium. He mentioned that besides the exogenous factors an endogenous factor probably also played an important role. The possibility that radon may be of importance in the cancer of the lungs of the miners had been expressed previously, e. g., by Ludewig and Lorenser (*18*) in 1924. At that time the carcinogenic action of X or γ radiations had been known for many years.

In 1935 Lange (*8*) stated that the mortality in Schneeberg caused by cancer of the lung was still 60 to 70 percent of all deaths of miners or pensioners. He could not find silicosis in the miners he checked, and he believed that perhaps not radon but radium, besides the carcinogens, arsenic and cobalt, was to blame for the high incidence of lung cancer. He determined on a map the location of 266 deaths from cancer and stated that cancer was 3 times higher in districts rich in radium than in regions with less radium.

In 1936 the German committee for the fight against cancer started a detailed investigation in the Schneeberg region. In the following year Brandt (*11*) reported on the program, which consisted of clinical investigations, necropsies on all miners, statistical investigations, measurements of radioactivity, and animal experiments. The results of the anatomical investigations were reported by Hueck (*5*) in 1939. From 1936 to 1939, of 70 miners, 6 died and were necropsied, 5 showed carcinoma of the lung, and 1 tuberculosis. Necropsies on 14 persons in Schneeberg and surrounding country (it is not stated whether

they were miners) showed 12 cases of carcinoma of the lung. Hueck stated that the lung cancer of miners was not different from that of other people. Silicosis had no immediate connection, as the persons dying from silicosis showed no cancer while often those with cancer of the lung showed little silicosis.

Lange (*8*) pointed out that since the establishment of a cancer consultation center in the region, the number of people found with pulmonary cancer had increased. No statistics were given to indicate whether the incidence of this type of cancer is generally high in this region. He stated that the radioactivity in rock and air was undoubtedly the cause.

Until the time of the investigations of Rostoski, Saupe, and Schmorl (*1*) in Schneeberg lung cancer was unknown among the miners of Joachimsthal (*4, 15*). In the mortality statistics tuberculosis was frequently given as cause of death. As a result of the findings in Schneeberg, investigations were started in Joachimsthal, and in 1929 Löwy (*6*) reported one case in which a lung tumor was found in a miner by bronchography and another of a worker in the radium factory, who showed a lung tumor at necropsy. Histologically, the tumor proved to be an undifferentiated carcinoma.

This first investigation was followed by others in rapid succession. In 1930 Šikl (*2*) reported necropsy findings on 15 miners and pensioners. Eight had cancer of the lung with either squamous-cell carcinoma or small-cell carcinoma. These miners, whose ages ranged from 40 to 63 years, had worked in the mines for 13 to 23 years. A chemical analysis of the lung parenchyma showed calcium, magnesium, aluminum, and silica. No radioactivity was found. Šikl assumed that radon was the cause of the neoplasms. In 1930 Ziel (*12*) reviewed the findings up to that time and stated that of a total of 322 miners, 60 percent showed pneumoconiosis. Anamnesis showed 47 cases of cancer in their parents or brothers and sisters. No cancer was found in active miners. In 1932 Pirchan and Šikl (*19*) reported a detailed investigation; 323 miners were given a thorough physical and fluoroscopic examination. No cases of lung cancer were found. Of 83 retired miners the diagnosis of cancer of the lung was made in 3 (2 were later confirmed by necropsy). Between 1929 and 1930, 19 miners died; 13 of them were necropsied, and 9 showed cancer of the lung. Six of the miners had been retired 6 to 27 years before examination. The authors believed that cancer of the lung developed more rapidly in Joachimsthal than in Schneeberg. They also pointed out that pneumoconiosis was much less severe in Joachimsthal than in the Schneeberg miners and not important for the development of cancer. Teleky (*20, 21*), however, believed that the differences in pneumoconiosis between Joachimsthal and Schneeberg might be due to the technique of examination employed (in Joachimsthal probably fluoroscopy only was used, while radiographs were taken in Schneeberg). Pirchan and Šikl (*19*) also reported on the chemical analyses of lungs. No radium, calcium, magnesium, silica, arsenic, bismuth, cobalt, or nickel was found. They stated that radioactivity was to blame and mentioned that the miners believe "that the discovery of a rich uranium ore vein is always followed some years later by a strong mortality among them." Woldrich (*22*) examined the blood of 29 pensioned miners, active miners, and workers in the uranium factory. He found anemia in 23, especially in factory and laboratory workers, and stated that this was "myelotoxic enemia due to radium or radon inhalation." As only single blood examinations were made, his conclusions have little significance. In 1935 Ziel (*23, 24*) reported on 15 necropsies in Joachimsthal, of which 4 showed cancer of the lung. Chemical analysis of lung tissue showed no lead, arsenic, bismuth, cobalt, nickel, or uranium, but considerable silica. In miners with tuberculosis also a high silica content was found. In 1935 Tschelnitz (*25*) stated that radon was not the sole cause

of the cancer of the lungs in Joachimsthal and believed that the most likely cause was the radium inhaled with the dust and the pneumoconiosis produced by the inhalation of silica. In 1939 Peller (*26*) summed up the necropsy findings in Joachimsthal from 1928 to 1938. Of 89 miners and pensioners who died from 1928 to 1938, 60 were necropsied. Of these 43, or about 50 percent, had cancer of the lungs. Of 30 who died from other causes, 24 died of lung diseases, and of these 18 had tuberculosis (4 combined with silicosis) 3 severe silicosis without tuberculosis, 1 emphysema, 1 pneumonia, and 1 chronic bronchitis. In 1939 Saupe (*27*) made an X-ray investigation of 398 miners or workers in the radium factory and pensioners with 10 years or longer activity. His findings are given in table 1. The silicosis found was the same as that observed in stone workers or in miners generally. The radium factory also was not free of dust. Two of the three men with definite lung tumors had not been in the mines for 12 and 20 years, respectively. There were few miners who did not complain of cough, chest pain, or dyspnea. Since 1939 no additional information has been published. The available data show that approximately 50 percent of all miners of Schneeberg and Joachimsthal dying up to 1939, died of cancer of the lung. The cancer, formerly diagnosed as lymphosarcoma, was later diagnosed in all cases as carcinoma. Whereas in former years pneumoconiosis in combination with arsenic and cobalt was assumed to be the cause of this cancer, today the radioactivity of the ore and the radon content of the air in the mines are generally considered to be the primary cause.

TABLE 1.—*X-ray findings in miners*

X-ray diagnosis of lungs	Miners	
	Number	*Percent*
Well	216	54.1
Silicosis I	134	33.6
Silicosis II	28	7.1
Silicosis III	4	1.1
Silicosis plus advanced tuberculosis	6	1.6
Advanced tuberculosis without extensive silicosis	3	.7
Pulmonary cancer	3	.7
Suspicion of pulmonary cancer	4	1.1

PHYSICAL DATA

The region of Schneeberg and Joachimsthal is rich in radioactive substances. However, the radium content of the rocks in both mining districts and in the mines is relatively low, approximately 2×10^{-11} gm. radium per gram of rock in comparison with 2×10^{-12} gm. radium in granite. It is due to impregnation of the rock with finely dispersed uranium minerals. In Joachimsthal pitchblende is obtained in some mines. There the pitchblende is admixed with many other compounds, especially lead sulfide, silica, calcium oxide, ferrous oxide, and magnesium oxide (Hevesy and Paneth (*28*)). No data were found as to the content of pitchblende in the mined ore. The average total radium output in Joachimsthal is approximately 2 gm. per year. Ten thousand kilograms of pitchblende yields approximately 2 gm. of radium. As shown later, the radon content of the air and water in the mines and springs (some of which are thermal springs) is considerably higher than corresponds to the radium content of the rock. It is assumed that the radon comes from radium-containing ore at great depth and diffuses into the mines or is carried up by the springs (*3*). The first detailed physical investigations in Schneeberg were carried out by Ludewig and Lorenser (*18*). They found that the radon content of the air in the mines varied from 3.6×10^{-10} to 1.8×10^{-8} curies per liter, most values were below 3.6×10^{-9} curies. The highest values were encountered at places where the air was stagnant, especially at the places of boring operations. In one mine, "Siebenschlehen" (called by the miners the death mine), the radon content was between 3.6×10^{-9} and 9×10^{-9} curies per liter. Between 1936 and 1939 Rajewsky (*10*) made exhaustive measurements. He not only investi-

gated the radon content of the air in the mines but also measured the amount of radon in the exhaled air and in the urine of miners. The radon content of the air in mines varied from 7×10^{-12} to 7×10^{-9} curies per liter and was in most cases below 3.6×10^{-9} curies. In the mine "Siebenschlehen," which was abandoned since the measurements of Ludewig and Lorenser were made and became flooded subsequently, he found values up to 5.4×10^{-8} curies per liter. The higher value measured after the mine was abandoned was undoubtedly caused by the accumulation of radioactive water and stagnation of air. In the mines in operation at the time of measurement, the radon content was between 2 and 3×10^{-9} curies per liter. The springs in the mines showed a radon content similar to that of the air.

The data on the exhaled air of miners were as follows: Before entering the mine shaft, 4×10^{-10} to 1×10^{-9} curies per liter; after leaving the mine shaft, 9×10^{-10} to 1.8×10^{-9} curies per liter; on leaving the mine house, 5 meters from the mine house 1×10^{-11} to 8×10^{-10} per liter; and 100 meters from the mine house, 7×10^{-11} to 2×10^{-10} curies per liter. In another group the radon content was 0 to 7×10^{-11} curies per liter before entering and 0 to 4×10^{-11} when leaving the mine. In Joachimsthal, Běhounek (*29*) investigated the radon content of the mines from 1924 to 1925. He found values similar to those found in Schneeberg by Ludewig and Lorenser and by Rajewsky. The air in the mines had a radon content of 4×10^{-10} to 2×10^{-8} curies per liter, most values being under 4×10^{-9}. In two drilling holes he found activities up to 8×10^{-8} curies per liter. The activity of the springs in mines ranged from 1×10^{-9} to 2×10^{-6} curies per liter. The majority of the springs, however, had an activity considerably below 4×10^{-8} curies per liter.

According to Rajewsky (*30*), the human body contains approximately 1×10^{-8} gm. of radium in middle age. Of this amount, 90 percent is deposited in the bones, and 10 percent in blood, lung, liver, spleen, etc. The smallest amount is found in the muscles. The radium content of the lungs varies from 0.05 to 2 if the content of the vertebrae is 100. It is approximately $\leqq 2 \times 10^{-13}$ gm. of radium per gram of fresh lung tissue. In 1934 Běhounek (*31*) reported on the radium content of lung tissue and vertebrae of miners of Joachimsthal who had died of cancer of the lung. He found approximately 1×10^{-12} gm. of radium per gram of lung tissue and approximately the same amount per gram of vertebra.

Rajewsky (*10*) made measurements on lung tissue of 15 miners (41 to 64 years old), who had died of cancer of the lung and found values ranging, according to age, from 0.06×10^{-12} gm. radium equivalent to 2×10^{-12} gm. radium equivalent per gram of fresh lung tissue, the latter value in a miner 61 years of age. The majority of his values were below 0.5×10^{-12} gm. radium equivalent. Data for vertebra (two cases) were approximately 1×10^{-12} per gram of fresh bone, and for muscle 0.06×10^{-12} gm. radium equivalent per gram of fresh tissue. These values are not different from the data given for the average human being of middle age, considering individual variations and the large error connected with measurements of such small activities. If large amounts of radon are inhaled constantly, the amount of the long-life decay products accumulated is so small that they fall well below the range of the natural activity of the body. Fernau and Smereker (*32*) calculated that in the oral administration of 3.6×10^{-6} curies of radon daily over a year the total accumulation of radium D would amount to 5.06×10^{-12} gm., that of radium E to 3.13×10^{-15} gm., and that of radium F (polonium) to 8.23×10^{-14} gm. Therefore, the activity measured in tissue is in no way a measure of the amount of radon inhaled previously.

In order to determine a toxic dose for radon inhalation over long periods of time, Rajewsky (*10*) calculated this dose from the data of Inouye (*33*) on the decrease of anaerobic glycolysis of sarcoma tissue under the influence of radon.

A 10-percent decrease in the value of glycolysis, assumed to be the lower threshold value for a toxic dose, is produced by 1.5×10^{-5} curie-hours per gram of tissue. This dose compares with the lower limit of the toxic dose for radium (calculated from a lethal case of radium poisoning) of 2.6×10^{-5} gram-hours of radium element per gram of tissue. Assuming that the miners work on an average 18 years and spend 8 hours daily in the mines, and considering further that it takes 2 to 3 hours until the emanation is removed from the body and the fact that long-life products are deposited at first in the lungs, Rajewsky obtained from the foregoing tissue value a value for the lower limit of the toxic dose of inhaled radon of 9.5×10^{-8} curies per liter of air. The tolerance doses should be $\frac{1}{10}$ to $\frac{1}{100}$ of this dose, approximately 1×10^{-8} to 1×10^{-9} curies per liter of air. It has been shown that the air of the mines contains an average of about 3×10^{-9} curies per liter of air. Rajewsky concluded—

> that the possibility that the damage produced by the inhalation of the air of the mines containing radon is *one* of the causes of the disease of Schneeberg cannot be denied on the basis of the measurements at present available.

In 1939 Read and Mottram (*34*) arrived at a value for the safety dose in another way. By exposing mice to radon of different concentrations, they found that a concentration of radon of 5×10^{-7} curies per liter is fatal to mice within a few weeks, and that mice will live 161 days (the time of termination of the experiment) in an atmosphere of 1×10^{-7} curies per liter. At this concentration the animals breed and grow normally. As safety dose they propose one-tenth of this value, 1×10^{-8} curies per liter. This is in agreement with the data of Rajewsky. In the United States Evans and Goodman (*35*) in 1940 proposed a safety dose of 1×10^{-11} curies per liter for continuous inhalation of radon. They argued that in the Schneeberg and Joachimsthal miners the average radon concentration is 2.9×10^{-9} curies per liter. This dose over long periods of time, they said, will produce lung tumors in the miners. On the other hand, one microgram of stored radium in the body will produce 1.1×10^{-11} curies per liter of exhaled air on an average. In the case of one dial painter with 2 micrograms of stored radium,[1] carcinoma of the ethmoids and antrum was found.[2] This painter exhaled continuously 2×10^{-11} curies of radon per liter. Hence, Evans proposed a safety dose of 1×10^{-11} curies of radon per liter. This dose has been adopted generally in the United States as the safety dose.

ANIMAL EXPERIMENTS

As mentioned previously, the belief is growing that the continuous inhalation of radon is responsible for lung cancer in the miners of Schneeberg and Joachimsthal. The question arises: How far do animal experiments support this belief? A considerable number of investigations have been carried out on the biologic effects of radon. As most of them are short-term experiments and as the induction of lung tumors by radon is not reported, they are omitted here. They are reviewed by Stoklasa (*3*). In the following discussion only prolonged experiments or experiments using material from the mines (such as dust, etc.) are considered. The first animal experiments were made by Härting and Hesse (*4*), who fed fungi from the mines to mice. Arnstein (*13*) fed mud from the mines to white rats and injected cultures of molds from the mines into rats intratracheally. The basis for these experiments was the idea that a gaseous product of arsenic might be formed by the fungi. These experiments were negative. Schmidtmann (*36*) in 1930 exposed animals to six different samples of boring dust from the Schneeberg mines. He could not obtain dust collections in the lungs characteristic of those found in the Schneeberg miners. The action of the different dust samples on

[1] Personal communication to Evans (*35*) from Martland.

[2] No evidence is cited that this was not a purely coincidental spontaneous neoplasm.

the bronchial epithelium varied, two dusts containing the most arsenic regularly produced peribronchitis with strong connective tissue reactions and epithelial proliferation. Smiliar changes could be produced with other dusts if small amounts of arsenic were added to the food. However, he stated that arsenic could not be the only or essential agent in the dust, as street dust and arsenic never produced such changes. According to Teleky (*22*), unsuccessful animal experiments were carried out by Löwy (*37*) in 1936 in Joachimsthal.

An extensive experiment, in which animals, mainly mice, were placed in the mines in the neighborhood of the working place of the miners, was started in Schneeberg in 1936 by Döhnert (*9*). He stated that white mice of both sexes were used and were procured from a dealer who had the greatest interest in having tumor-free colonies. No controls were kept. At the beginning of the experiment the animals were over 1 year old, because Döhnert reasoned that older animals get tumors more easily. The reasoning for this experimental setup seems very strange, especially since the importance of the use of inbred mice with high spontaneous-tumor incidence (e. g., strain A mice with 50-percent incidence of spontaneous lung tumor at the age of 12 months) in cancer research has been known and stressed for many years. No conclusions can be drawn from reports in the literature on the incidence of spontaneous lung tumors in stock white mice as to the incidence in any given batch of white mice. The incidence may change considerably from batch to batch. Furthermore, in Döhnert's work only dead mice came to necropsy. All these factors make his results and conclusions of questionable significance. They are given, however, because he thought they showed that the radioactivity of the mines was mainly responsible for the cancer of the lungs of the miners. He stated that on account of the given considerations the content of radium of the Schneeberg mines must be considered as a carcinogenic agent of decisive importance. He reported that 28 mice were exposed in the mines from 2 to 370 days. Almost all the animals died of infections of the respiratory tract (bronchitis or pneumonia). Moderate dust deposits were observed in the lungs. Twenty-five percent of the animals had tumors. Two were epithelial lung tumors, one a focus of hyperplasia of lung (perhaps due to coccidiosis) the remaining tumors were at other sites. He assumed that the percentage of lung tumors was considerably increased in comparison with Slye's 2.7 percent and Schabad's 3.5 percent for white mice. He considered also the total tumor incidence of 25 percent far above the normal. Discarding a possible carcinogenic action of arsenic and also possible effects due to the climate in the mines, he concluded that the radioactivity of the mines was the only factor that caused the induction of tumors in his experimental animals.

In 1939 Hueck (*5*) reported on the continuation of Döhnert's experiments. Of 110 mice which had died up to that time, 18 mice had tumors, 5 having adenomas of the lungs. Fifty-two of these animals were given to Rajewsky for analysis of the radium content. Rajewsky (*10*) gave the following values per gram fresh tissue of 15 animals: bone and marrow from 1.4 to 75 x 10^{-12} gm. radium equivalent, and lungs from 0.5 to 18.2 gm. radium equivalent. In conclusion it can be stated that the animal experiments carried out in the mines or with material obtained from the mines do not offer any proof that the radioactivity in the mines is responsible for the cancer of the lung of the miners.

A number of experiments with radon under laboratory conditions lasting from a few weeks to over a year are reported in the literature. In 1926 Jansen and Schultzer (*38*) kept young and suckling rats in an emanatorium containing in one experiment 7.3 x 10^{-7} curies per liter, and in another 1.8 x 10^{-7} curies per liter. The rats of the first experiment became sick and lost weight after 5 weeks and were moribund or died 1 to 2 weeks later. At necropsy,

hemorrhages in the lungs were observed. Exposure to 1.8 x 10^{-7} curies per liter had no demonstrable effect after 9 weeks. Schultzer (*39*) implanted paraffin capsules with 0.040 to 0.200 gm. of a radium bromide solution of 15 to 20 mg. percent in the abdominal cavity of rats. The radon diffused from the capsule into the rat. Schultzer found the radon content of the animals was approximately the same as that in the experiment of Jansen and Schultzer, in which the animals lived in an atmosphere containing 7.3 x 10^{-7} curies of radon per liter. In contrast with the emanatorium experiment, the rats showed no ill effects after 9 weeks. Schultzer concluded that the rats in the emanatorium experiment dying after 7 weeks of exposure succumbed, not on account of the radiation from the radon, but probably on account of the γ radiation originating from the decomposition products within the emanatorium. No lesions of the lung were observed.

Stoklasa (*40*) in 1932 exposed rabbits 6 to 8 hours per day for a total of 120 days to an atmosphere containing 2.9 x 10^{-8} curies per liter. A few animals died after 65 to 90 days. He repeated the experiment and analyzed the lungs for lactic acid and enzymes and found an increase in lactic acid. He assumed the increase to be due to an increase in the breathing process resulting in straining and damage of the lungs, and finally in pathologic changes. He stated that the problem of the lung cancer of the miners of Joachimsthal seemed to be solved by his experiment. No pathologic data were presented to support such a speculation.

In 1938 Lorenz[3] injected 11 male strain A backcross mice, 3 months of age, intraperitoneally with a radon solution in water 4 times within 2 months with the following doses: 0.2, 0.22, 0.4 and 0.38 millicurie, a total of 1.2 millicuries. Up to the fourth dose the animals were in good physical condition, but afterwards their weight and health began to decline. Fifty percent of the animals had died or were killed and necropsied when in a moribund condition 6 months after the first injection. The remainder lived to about 8½ months after the first injection and were killed and necropsied also when in a moribund condition. The decline of weight was rapid during the last month, the fur became gray generally and white over the thorax. Necropsy showed extreme atrophy of spleen, bone marrow, lymph nodes, and sex organs. No increase in lung tumors was observed in comparison with controls, nor was there any damage to the lung tissue although at least 90 percent of the radon is excreted through the lungs. Strain A backcross mice show a high incidence of spontaneous lung tumors, and lung tumors are readily induced in them with carcinogenic compounds.

The experiments of Read and Mottram (*34*) were mentioned in the preceding section. Mice exposed to a concentration of 1 x 10^{-6} curies per liter for 26 days (except Saturdays and Sundays) began to die off after the twenty-second day. The histologic findings were similar to those in animals exposed to γ radiation or X-rays; i. e., general atrophy of lymphoid tissue, a bone marrow containing chiefly adult polymorphs, ovaries showing degenerated and shrunken ova, and atrophy of testes. No radiation damage to the lungs was mentioned. Animals exposed to 5 x 10^{-7} curies per liter for 43 days (only 1 surviving the forty-eighth day) showed similar histologic findings. The authors state: "The only additional pathologic finding was an abscess of the lung in one animal." Mice exposed to 1 x 10^{-7} curies per liter over a period of 161 days showed normal growth and breeding.

Jackson (*41*) exposed male mice of strains C57 Black, A, and C3H (a total of 61 mice) continuously to an atmosphere of 1.5 x 10^{-6} curies per liter. After 60 days, 13 mice had died of intercurrent disease, no lung changes were reported in these animals that could be attributed to direct radiation damage of the lungs.

In 1943 Rajewsky, Schraub, and

[3] Unpublished data.

Kahlau (*42*) reported in a preliminary communication experiments in which white mice were exposed continuously to radon. In 1 experiment (36 animals) the following concentrations were used: 1.4×10^{-5}, 1.5×10^{-5}, and 2.0×10^{-5} curies per liter. These doses proved toxic, and the animals died within 60 to 100 days. Rapid decrease in weight and pathologic changes in the blood picture were observed. Histologically atypical changes of the bronchial epithelium were found which increased with increasing length of exposure and were characterized by irregular arrangement and appearance of abnormally large cells. In another experiment, white mice were exposed to 1.16×10^{-6} curies per liter. In this concentration the animals lived from 161 to 453 days. A decrease in weight was observed after 83 days (from 19.4 to 15.4 gm.) and the animals died in a pronounced sick state. Histologically, the atypical changes of the bronchial epithelium were also observed in this series. In the 12 experimental animals, 10 adenomas, 1 adenocarcinoma originating from a small bronchus, and 1 peribronchial and perivascular proliferation of the lymphoreticular tissues were observed. In the control series only 1 small adenoma was found. The authors stated that there exists hardly a doubt that the described findings, 58 percent of the tumor animals, 10 adenomas and at least 1 bronchial carcinoma in 12 animals must be attributed to the chronic action of the radium emanation.

The objection to the use of stock white mice in cancer research has already been raised. The weight of the mice was 19.3 gm. after 83 days indicating that either very young animals were used and their growth was stunted from the beginning of the experiment or that the animals became sick at an early stage. Mice having chronic pneumonia may live for many months with their weight slowly declining. Of the 12 experimental animals, 7 showed 10 adenomas and 1 adenocarcinoma originating from a small bronchus. There is no convincing evidence in the literature that spontaneous adenomas or adenocarcinoma of the mouse lung originate from bronchial epithelium (*43*). All evidence points to an alveolar origin of these tumors. In all experiments in which carcinogenic chemicals were used for the induction of lung tumors, by either the subcutaneous or the intravenous (Andervont and Shimkin (*44*)) or the intratracheal (Shimkin (*45*)) route, only adenomas of the alveoli were induced. Radon, if carcinogenic, should also induce tumors of the alveoli mainly unless the mechanism of tumor induction by radon is different from that by chemical carcinogens. Rajewsky et al. did not give the location of the adenomas. As they stressed the fact that 1 adenocarcinoma developed from a small bronchus and that the bronchial epithelium was strongly atypical, however, it is believed that the adenomas also developed from the bronchi. It is a well-established fact that chronic pneumonia in mice very frequently shows bronchiolar proliferation, which, when occurring as a localized nodule, resembles an adenoma. Such changes were described in detail in mice and other small laboratory animals (*46, p. 54*). The question arises whether the adenomas observed by Rajewsky et al. were not such structures. The body weight of the experimental animals, as discussed herein, supports such a theory. Mice kept in an enclosed space continuously or for a number of hours per day for long periods are prone to develop chronic pneumonia unless humidity in the space is controlled.[4] It seems, therefore, that as far as this preliminary report is concerned, the proof that radon can induce tumors of the lung of mice is still lacking. The conclusion is reached from the experimental material presented that up to the present time it is doubtful whether radon can induce lung tumors in experimental animals.

DISCUSSION

In the course of time, various factors have been blamed for the high incidence

[4] Lorenz: Unpublished observations.

of lung cancer among the miners of Schneeberg and Joachimsthal. While in the early stages of the investigations, poisonous factors in the mines, such as fungi or the mud, besides dust containing arsenic, cobalt, and silica, were thought to be the cause, attention shifted in the early twenties mainly to the dust, the pneumoconiosis connected with it, and chronic diseases of the respiratory tract. At that time, however, the opinion had already been expressed that the radioactivity of the mines might be a contributing cause. In later years, it became dubious (cf. Macklin and Macklin (*47*)) that chronic irritation is the cause of primary carcinoma of the lung. No report has been found in the literature that arsenic, although inducing other lesion, after long contact will induce pulmonary cancer (*48*). Therefore, these factors must be excluded as primary factors. With the increasing knowledge of the danger of radioactive material to biologic matter and with the knowledge that radiations can induce carcinoma of the skin, and radium, if lodged in the bones, will induce bone sarcomas (Martland (*49*)), attention became more and more centered on the radon content of the mines. Finally radon was considered as the sole cause of the cancer of the lungs of the miners.

Isolated opinions were still voiced at that time about other possible causes. Dreyfus (*50*) in 1936 described two cases of carcinoma of the lungs in persons that as children had inhaled iron dust for 12 years, whereas other children in the same family not living in the atmosphere of dust were free from it. He pointed out that this finding may be of importance in the genesis of lung cancer in miners. Rostoski, Saupe, and Schmorl (*1*), later Döhnert (*9*), and Brandt (*11*) pointed out that an endogenous factor may play a role. Evans and Goodman (*35*) after calculating the α-ray dosage for bone cancer and the α-ray dosage to which the lungs of the miners are exposed to 4.6 r ergs per hour per gram of dry bone and to 0.17 erg per hour per gram of dry lung tissue, state:

> Lung tissue appears to be about 25 times more susceptible to radiation than bone tissue . . . and we know that 4.6 and 0.17 erg units over extended exposures will produce cancer in bone and lung tissue respectively.

Reports in the literature on cancer of the lung of workers in radium laboratories seem to support this point of view. Neitzel (*51*) described two cases of carcinoma of the lung in workers in radium laboratories. He also mentioned Löwy's two cases of workers in the radium factory of Joachimsthal who died of cancer of the lung. The workers had never been employed in the mines. It is difficult to evaluate the significance of these isolated cases, as no statistics are available on the number of people engaged in work with radioactive substances.

In the discussion of a paper by Maisin (*52*) in 1934, De Laet stated that in Belgium radium is prepared for almost the entire world and that no cancer of the lung had been observed in the radium plant in 12 years of his experience. The workmen are literally covered with dust and absorb it all the time especially by mouth. They are yellow from head to foot and will not wear masks. Maisin, in answering De Laet remarked that 12 years is not long enough to permit judgment. He believed that some would eventually develop cancer. Tschelnitz (*25*) cited experiments in which people drank water containing 3.6×10^{-4} curies of radon daily for 2 months without ill effects. Lange (*8*) in 1935 stated that no cancer of the lung had been observed in workers in the radioactive bath of Oberschlema in Saxony, in which water containing from 1×10^{-8} to 2×10^{-6} curies per liter is used. However, the radioactive baths had been used for only 8 years. As proof that radon is not the cause of lung tumor Martland's (*49*) investigations have been cited of the luminous-dial painters who developed only bone sarcoma although they exhaled radon continuously. According to Evans and Goodman (*35*), 1 microgram of stored radium produces a concentration of 1.1×10^{-11} curies of radon

per liter of exhaled air. This concentration is too low to have any possible effects on the lungs, even if the amount of stored radium is considerably higher. It may be seen, therefore, that there is no clear-cut evidence that radium or radon have ever induced lung cancer in man outside the mining district of Schneeberg and Joachimsthal.

Little is known of nonmalignant damage to lung tissue by radioactive substances. Doenecke and Belt (*53*) described the case of a chemist in a luminous-paint factory who after 4 years' work died from almost complete fibrosis of the lungs. However, his blood picture and bone marrow were normal, and no radioactivity was found in the lung tissue. The chemist blamed inhalation of radon for his disease.

On the other hand, damage to lung tissue by X or γ radiation is well-known. The literature on this subject has been reviewed by Warren (*54*) and may be summarized as follows: The lungs should be classed as a moderately sensitive structure; heavy doses (1,000 to 10,000 r) will cause transient radiation pneumonitis of a severity varying with dose. Severe necrosis of the lungs occurs 2 to 3 weeks after exposure from 15,000 to 21,000 r (given in five or seven exposures) in rabbits. Fibrosis, often present, has not been established as a radiation effect independent of intercurrent infection. There is a close parallel between the changes induced by these radiations in man and in animals. There is no report in the literature that carcinoma of the lung has been observed following radiation damage to the lung by X- or γ rays.

It can be argued that the biologic effects of α rays and X-rays are different. Absorption of α rays or X-rays results in the formation of ion pairs, the total number of ion pairs per particle being dependent on the energy of the particle and the energy necessary for the formation of an ion pair, approximately the same for both radiations. Therefore, for equal absorbed energy there can exist only a quantitative difference in the biologic action of radiation if, for example, the time in which the dose is given is varied from one experiment to another. Assume an experiment in which per unit time the same amount of either α radiation or X-rays is absorbed per unit volume, then the biologic effects must be identical. Blum (*55*) found that the tumor induction time by ultraviolet radiation is independent of the intensity for the same daily dose given 5 days every week to a certain lower limit. Below this limit of intensity the tumor induction time increases rapidly. In other words, beyond a certain dilution of the dose the effectiveness of the agent decreases rapidly, no matter how long the agent is applied. The same holds true for other radiations, beyond a certain limit of intensity the biologic effects of radiations diminish for the same total dose. These limits of intensity are different for different radiations and probably are considerably lower for α rays than X-rays as the density of ion pairs along the path of an α particule is much greater than for the path of the secondary electron produced by an X-ray photon. Without this dilution effect of the dose, the establishment of a safety dose would be impossible. The safety dose for X-ray is 10^{-5} r per second, which corresponds to an absorbed energy per cubic centimeter of tissue per second of 10^{-3} erg; 10^{-3} erg per cubic centimeter of tissue per second would be produced by about 6×10^{-10} gm. of radium per gram of tissue, which amounts to about 10 micrograms distributed in the bones of a man weighing 70 kg. It is known that this is above the radium deposits (up to several micrograms) found in man which will in some individuals induce bone sarcoma. In cases of radium poisoning the radium is not distributed evenly. Evans (*56, p. 1022*) states:

> The radioactive self-photographs of the bones of deceased victims show a lack of uniformity of distribution of radium. In some cases two or three small areas will be brilliantly self-photographic while the remainder of the bone will display only a moderate amount of fairly evenly distributed radiation.

This uneven distribution may well be the cause of the formation of bone sarcomas in spite of the small amount of total radium deposited. It should also be considered that in order to obtain a deposit of several micrograms in the bones, amounts up to several milligrams of radium may have to be taken. In the case of the luminous dial painters, this intake is distributed over many years. Therefore, it is difficult to compare the radium tolerance dose with that of the X-rays.

The radon dose corresponding to the X-ray safety dose was calculated by Read and Mottram (*34*) to be approximately 4×10^{-6} curies per liter of inhaled air. They found, however, that a dose of 5×10^{-7} curies per liter was fatal to mice within 43 days and that a dose of 1×10^{-7} was not fatal, a fact which indicated "that the tolerance concentration must be of the order of one hundred times smaller" than the dose calculated from the X-ray tolerance dose. This may indicate, contrary to X or γ radiation, a much more far-reaching accumulation of the biologic effects of the α radiation; it may mean also that the radon is not uniformly distributed over the tissue but stays in greatest concentration in the blood.

Summarizing, we find that large doses of X- or γ rays to the lungs (doses in which the total absorbed energy was above 10^5 ergs per cubic centimeter of tissue (1,000 r in air)) have not produced lung tumors. It seems, therefore, unlikely that the miners of Schneeberg or Joachimsthal exposed daily to a concentration of radon of 3×10^{-9} curies per liter for 20 years should develop lung cancer, as the total energy absorbed during that time per cubic centimeter of lung tissue amounted to only approximately 1×10^3 ergs. The energy absorbed in the lungs of the mice of Read and Mottram (*34*) during 161 days was of the same order of magnitude. No lung lesions were found in these mice. Certain strains of mice have a high susceptibility to spontaneous lung tumors and others low, but generally the statement of Slye, Holmes, and Wells (*57*) holds:

> It seems that man exhibits lung tumors exceptionally infrequently, and that mice show an even more exceptional prevalence of tumors of this organ.

Evans states:

> It will be noted from these data that rats and mice are about 40 times as resistive as humans to lesions caused by α rays when all dosages are expressed in erg units. The extrapolation of the results of animal experiments to man must recognize this great difference in susceptibility.

This statement seems not to be justified as far as the effect of radon on lung tissue is concerned.

The opinion that radon is the sole cause of the lung cancer of the miners therefore cannot be maintained. The contributing factors in the case of the miners which were already brought up in the different investigations discussed may include the following: Pneumoconiosis produced by the dust in the mines, chronic irritation caused by respiratory diseases, arsenic, radioactive substances, and perhaps a hereditary susceptibility. The mines have been operated for hundreds of years. As frequently is the case in Europe, the occupation of the father is taken over by the son; the miners live in isolated places, and inbreeding often occurs. All these factors may lead to a hereditary susceptibility. However, no statistical evidence on this point has ever been collected.

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RELATIONSHIP OF AMYLOID INFILTRATION AND RENAL DISEASE IN MICE

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INTRODUCTION

All who work with certain inbred strains of mice are aware of the frequency with which amyloid infiltration and renal disease may develop in the older animals. There is, however, very little published on the spontaneous occurrence of these conditions; and no record could be found which suggested a causative relation between amyloid infiltration and contracted or cystic kidneys. Yet these conditions need to be considered because they may affect many observations where inbred strains of mice are used; they are of interest in themselves, and they furnish examples for the study of degenerative processes which are little understood. In this paper, a form of spontaneous amyloidosis, which occurs particularly in strain A mice, is described, and the role that amyloid deposition in the kidney plays in the pathogenesis of a characteristic renal lesion of this strain is considered. Possible analogies to similar conditions in man are also pointed out.

Many reports are available on the occurrence of amyloid in man. The infiltration most often appears following some chronic suppurative process, particularly where bone or cartilage is involved; but amyloid may be found as an idiopathic or primary process, when no preceding disease is recognized. There are a number of studies on the experimental production of amyloid in the mouse, but very little has been written regarding its spontaneous appearance. While the literature on human nephritis is voluminous and a varied etiology is recognized, our observations indicate that the majority of the renal lesions in certain strains of mice are associated with amyloid infiltration. For this and other reasons, they cannot be compared with any common renal disease of man.

Kuczynski (*1*) in 1922 suggested a method for the experimental production of amyloid in the mouse by the injection of sodium caseinate. This method was utilized by a number of later workers, among them Jaffé (*2*), Smetana (*3*), and Grayzel et al. (*4*), who gave detailed accounts of the early deposition of this substance, and the final production of a severe amyloidosis. Of most interest in connection with the renal disease of mice is the observation of Smetana that the first trace of amyloid in the kidney was in the medulla near the papilla where it formed in the walls of the capillaries beneath the endothelial cells. The tubules later were surrounded by fine rims of amyloid and might finally be partly or entirely occluded. The glomeruli were relatively little involved. Jaffé also observed that macroscopically the kidneys showed the picture of the third (contracted) stage of amyloid nephrosis and that the surface was covered with coarse granules consisting of distended tubuli.

Kidney disease in mice was described briefly by Twort and Twort (*5*), who used the term "Klossiella nephritis" because of the usual association of an altered kidney and infestation with *Klossiella muris*. Gorer (*6*) gave the most complete available report on kidney lesions in pure lines of mice. He described first a metaplasia of Bowman's capsule, which Crabtree (*7*) proved to be normal for the mouse and to vary with the sex of the animal. Gorer described also a "hyaline disease" of the glomerulus, which resembled an amyloid infiltration, and a "cystic disease" of strain A mice that appears to be identical with the renal change so frequently found associated with amyloid infiltration in this strain.

MATERIAL

The material consisted of necropsy records from two inbred strains of mice, A and C3H, and one group of hybrids, ABC, and microscopic sections from these animals. Both strains A and C3H are highly inbred and have been used extensively in cancer research. The hybrids were obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, and were reported to be produced by the backcross to strain A of F_1 hybrids from A times other strains. A large number of sections, which had been fixed in Zenker-formol and stained with hematoxylin and eosin, were available from every animal. This material came from three different experiments which were in no way designed to demonstrate the development of amyloid; but since a number of both control and experimental animals had arrived at an advanced age, sections from them furnished excellent material for a study of degenerative processes. No difference in incidence of amyloidosis or renal disease was observed between the control and experimental groups. For this reason, the pathologic changes may be considered as of spontaneous occurrence.

The animals had received routine care and the usual diet. The sexes were about equally divided in strain A and the younger strain C3H animals. The 11 old strain C3H mice and all ABC hybrids were males. The type of mouse, number, ages, and the incidence of amyloid infiltration and renal disease are shown in table 1.

That amyloid infiltration generally precedes a characteristic renal disease is evident from table 1. Their incidence was closely parallel, amyloid infiltration appearing first. Only two strain A mice were noted with renal lesions in which amyloid could not be found in available sections. Among the ABC hybrids, many showed a severe degree of amyloidosis, and it was in this group that renal changes were found. The amyloid and renal changes reported in two strain C3H mice were minimum in degree and consisted of a single mass of amyloid in the endometrium and a small patch of interstitial scarring in the kidney.

TABLE 1.—*Incidence of amyloid and renal disease in mice*

Type of mouse	Number of mice	Age	Number of mice showing—		
			Amyloid	Renal disease	Both amyloid and renal disease
		Months			
Strain A	23	8–12	8	2	2
	76	12–16	51	43	41
Hybrid ABC	81	18–21	61	26	26
Strain C3H	122	8–12	1	1	0
	17	12–15	0	0	0
	11	16–18	0	0	0

Special stains for amyloid were made on formalin-fixed, frozen sections of tissue from old strain A mice suspected of having amyloid deposits. Iodine staining was usually negative, but in a few sections from the duodenum a deep-blue color developed after treatment with iodine and sulfuric acid. Methyl violet produced no metachromatic staining, but the suspected deposits took an intense bluish-purple color and stood out clearly from the lightly stained background. Congo-red staining gave a reddish-orange color which differentiated this material from other substances in the section. The English observers (Twort and Twort (*5*) and Gorer (*6*)) objected to calling this substance amyloid because of this variable staining. Amyloid in man is known to vary markedly in its staining behavior, and Hass, Huntington, and Krumdieck (*8*) observed that amyloid from the horse and rabbit gave the iodine reaction poorly. Whatever the actual nature of these deposits in the mouse may be, they are analogous to if not identical with amyloid, and this is the term used by American authors (Jaffé (*2*), Smetana (*3*), Grayzel et al. (*4*), and Wells (*9*)).

MICROSCOPIC OBSERVATIONS

Adequate descriptions of the appearance of amyloid when produced experimentally in mice have been given, but no special report on its spontaneous oc-

currence in laboratory mice has been found. Wells (*9, p. 472*) stated that he often observed it in the Slye mice, both in those with and without cancer, to a degree far beyond that ever described in human bodies, and that the readiness with which mice developed amyloidosis when not subjected to any experimental procedure threw doubt on the results obtained with them in the experimental production. The spontaneous disease seems to differ in certain important respects from the experimental and resembles in some particulars primary amyloidosis of man recently reviewed by Koletsky and Stecher (*10*). In the primary amyloidosis of man, the amyloid is found in mesodermal tissues, smooth and skeletal muscle, the cardiovascular system, and the gastrointestinal tract; whereas in secondary amyloidosis the organs usually affected are the spleen and liver. However, much overlapping in the two conditions may occur. In experimental amyloid of mice an early "crystalline" form is described, but this was not seen in our mice with spontaneous amyloidosis, and the infiltration was recognized as a homogeneous hyaline substance lightly stained with eosin. Involvement of the spleen and liver was seldom extensive. The areas in which the deposit was especially frequent are described herein.

The earliest and heaviest deposits of amyloid were usually found in the interstitial tissue of Brunner's glands in the duodenum (fig. 1). The submucosal tissue of the limiting ridge which separates the forestomach from the glandular stomach in the mouse was also a frequent site, even when other areas were unaffected. Infiltration in the colon was common, and the intestinal villi sometimes appeared like amyloid cores covered with mucosa.

Patchy areas of amyloid were frequent in the heart; and the larger pulmonary veins, which in the mouse are invested with myocardial fibers, often showed infiltration.

The veins, as just mentioned, often showed infiltration, but other parts of the lungs were usually free, except for occasional masses at the margins of the pulmonary lobes. Pulmonary tumors, which are frequent in strain A mice, sometimes showed a striking degree of infiltration, so that the histologic details of these tumors were obscured by the amyloid deposits. Ewing (*11, p. 30*) recorded infiltrations of amyloid in human tumors.

The other organs involved were the ovaries, testicles, adrenals, endometrial stroma, and skin. The deposit in the adrenal appeared to be on and around cells (fig. 2) and not interstitial, a point of amyloid deposition in man recently emphasized by Peters (*12*). In advanced cases, nearly every organ and tissue showed some deposits.

Amyloid infiltration in the kidney is described in the section following.

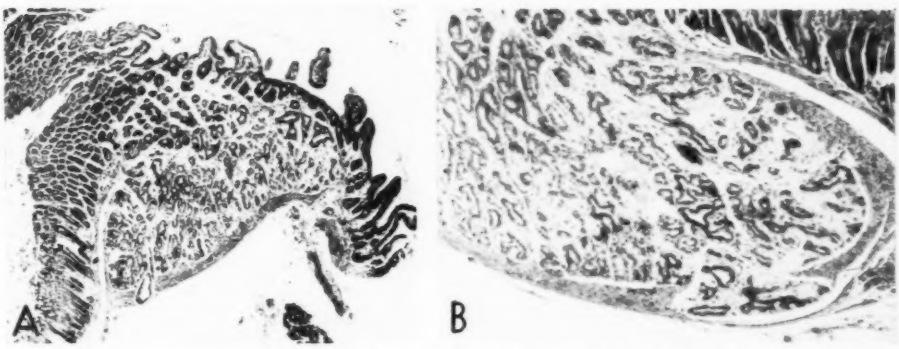

FIGURE 1.—Amyloid infiltration in interstitial tissue of Brunner's glands. *A*, × 30; *B*, × 65.

RENAL LESIONS

The changes in strain A mice followed a remarkably uniform pattern. They are described in detail herein, and modifications that appeared in other strains are noted.

The moderately advanced condition had many features resembling chronic or healed pyelonephritis of man. The glomeruli were generally unaltered; while an interstitial inflammatory reaction, colloid casts, periglomerular fibrosis, and exudate within the tubules, as mentioned by Weiss and Parker (*13*) in their description of human pyelonephritis, were usually found. The earliest microscopic change observed was a deposit of an amorphous, faintly acidophilic substance in the interstitial tissue between the collecting tubules of the papilla (fig. 3). This substance could be stained with Congo red, and the localization was similar to that given by Smetana (*3*) for amyloid. The next stage was necrosis of the tip of the papilla, similar to that described by Gorer (*6*), which may have resulted from interference with the blood supply in the long slender papilla of the kidney (fig. 4). The line of demarcation between the necrotic and living tissue was usually sharp, and calcium deposits often occurred, making a distinct calcium line (fig. 5, *A* and *B*). This necrotic tip was often sloughed off, an apparent dilatation of the pelvis developed, and the truncated base was some-

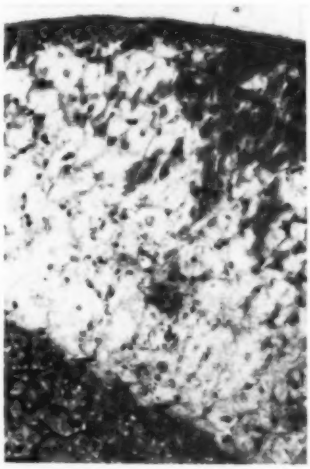

FIGURE 2.—Amyloid in the cortex of adrenal. The deposit appears to be on and around cells. × 150.

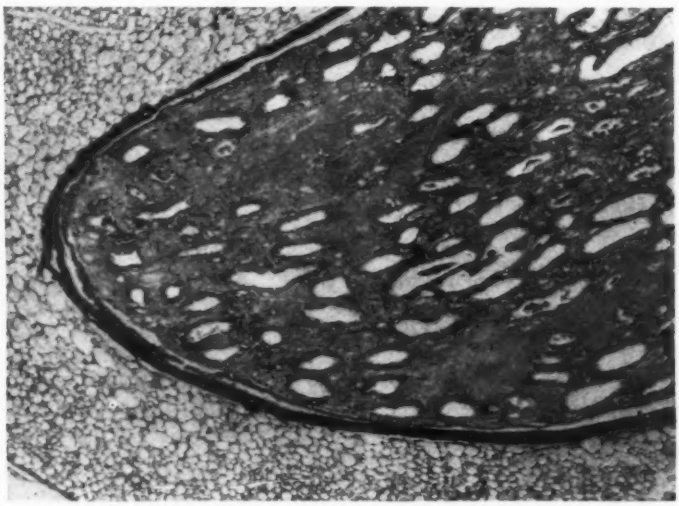

FIGURE 3.—Amyloid deposit in papilla of kidney, a stage preceding necrosis. × 70.

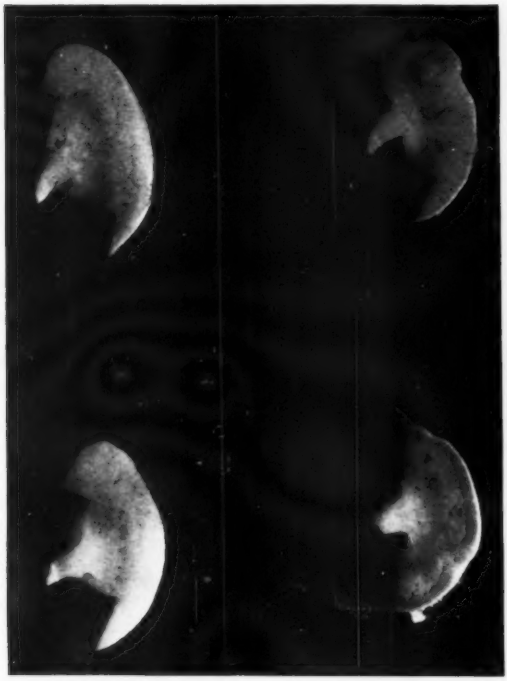

FIGURE 4.—Progression of the lesion in the papilla. Upper left kidney is normal. The first three kidneys are from strain A mice 12 months old, and the lower right kidney is from a mouse 20 months old.

times partially covered by pelvic epithelium (fig. 6, *A* and *B*). Calcification is not observed as a frequent complication of amyloidosis in man, and the calcium deposit here appeared to be distinct from the amyloid infiltration at the margin of necrotic tissue. No obstructions were found in the lower urinary tract, and the ureters did not appear thickened or dilated.

Changes in the cortex developed concurrently with the obstruction in the medulla. The convoluted tubules in well-delimited segments appeared first to be slightly and uniformly dilated, and at this time the surface of the kidney appeared to be mottled with light-brown translucent areas. The dilated tubules often contained an exudate of inflammatory cells, and it was not unusual to find multiple abscesses in the interstitial tissue at the time of death. It is not possible at present to say whether a stage of acute pyelonephritis with healing was necessary for the development of the final picture or whether the anatomical features of a healed or chronic pyelonephritis may have occurred without a definite acute stage. The most frequent condition of the tubules of the cortex in a moderately advanced stage was an apparent metaplasia in which a low columnar epithelium with rather basophilic cells replaced the acidophilic pyramidal cells of the normal proximal and distal convoluted tubules (fig. 7). Many of the tubules contained colloid casts, and thyroidlike areas could be found, a finding which Weiss and Parker (*13*) considered to be characteristic of healed or chronic pyelonephritis of the human

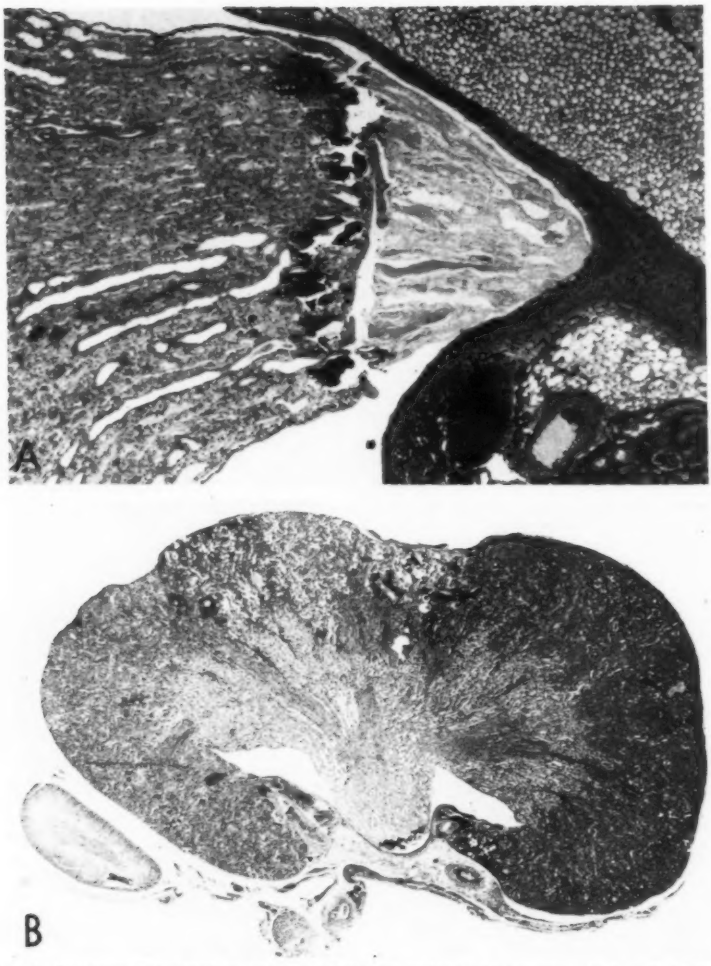

FIGURE 5.—*A*, Calcium deposited in papilla of kidney at line of necrosis. × 70; *B*. Early necrosis of tip of papilla of kidney, showing areas of contraction and dilatation of tubules. × 12.

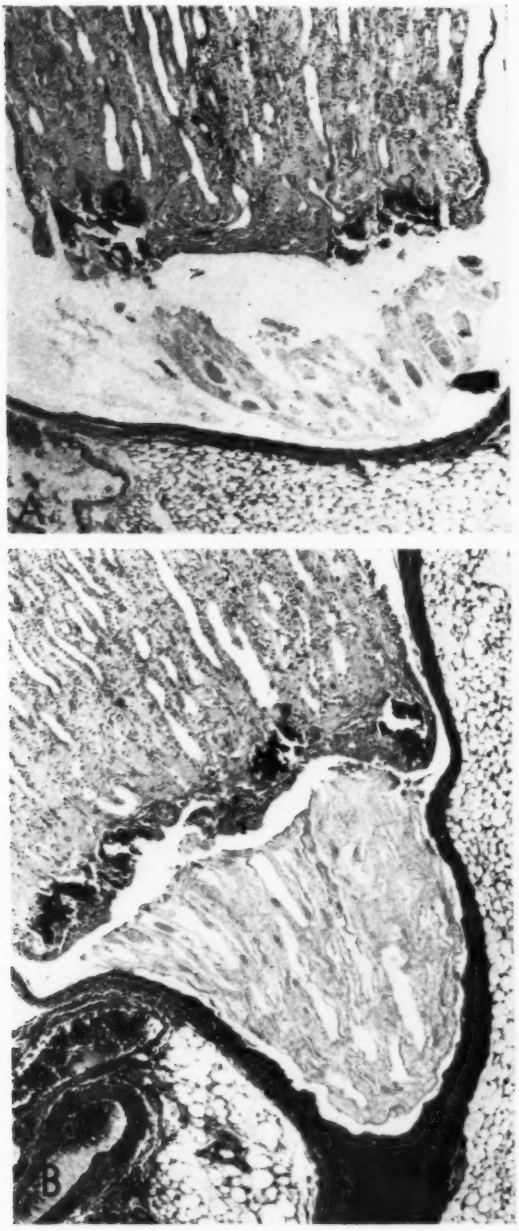

FIGURE 6.—A, Necrotic tip of papilla forming a slough in the kidney pelvis; B, Base of papilla partly covered by pelvic epithelium. Amyloid deposits can be seen above masses of calcium × 70.

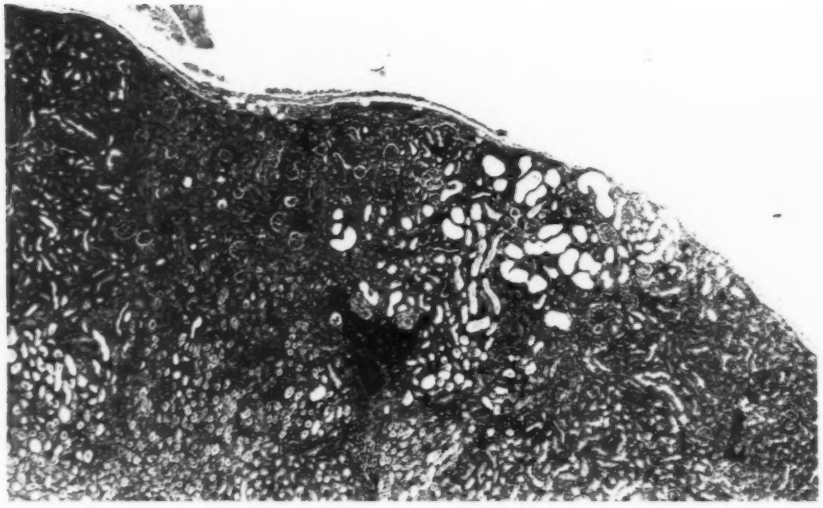

FIGURE 7.—Areas of tubular dilatation, apparent metaplasia, atrophy, and interstitial fibrosis in the cortex. × 30.

kidney (fig. 8). Interstitial fibrosis was apparent, and the kidney surface, on gross inspection appeared coarsely scarred (fig. 9). In the advanced stage, multiple large cystic dilatations often occurred in the cortex (fig. 10). Some of them appeared to originate as dilatations of Bowman's space, and the glomerulus could often be recognized in the smaller cysts, while a serous or mucoid fluid was found in the larger ones (fig. 11). This last feature appears to be peculiar to the mouse, and it may be owing to an anatomical difference in the species. The renal vessels showed no characteristic change; and although heavy lymphocytic infiltrations were often found in the larger arteries in early stages, the condition is observed so frequently in mice of many strains that no significance was attached to it.

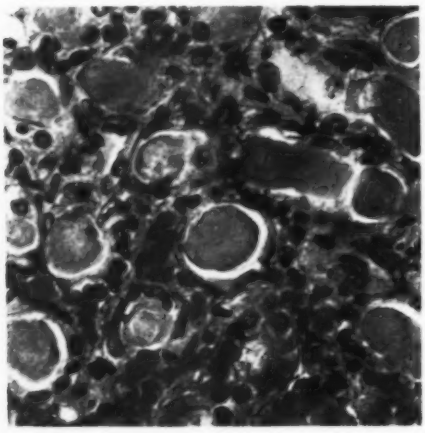

FIGURE 8.—Thyroidlike area containing colloid casts. × 150.

No measurements of the heart were made; and no microscopic myocardial change accompanying the renal lesions was recognized, although myocardial amyloid infiltration was usual and may have obscured other changes. In advanced stages, both the kidney and the liver appeared pale and brown; the general condition of the animals was poor.

The renal lesion in the ABC hybrids was generally similar, except that amyloid infiltration of the interstitial tissue of the papilla was more extensive, and necrosis and calcification near the tip of the papilla were less frequently observed. Interstitial deposits of amyloid in the cortex were also seen more frequently, and in a few instances a marked amyloid infiltration was observed in the glomeruli.

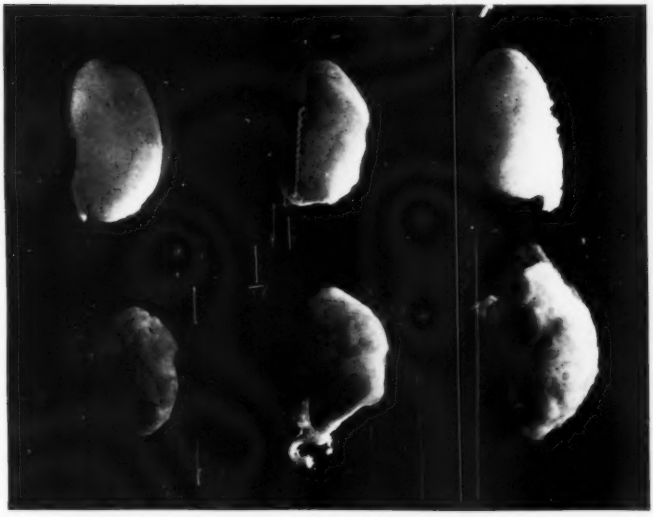

FIGURE 9.—Progression of the renal lesion. The upper three specimens are from strain A mice 12 months old; the lower three, from mice, 20 months old, show the cystic stage. × 3.

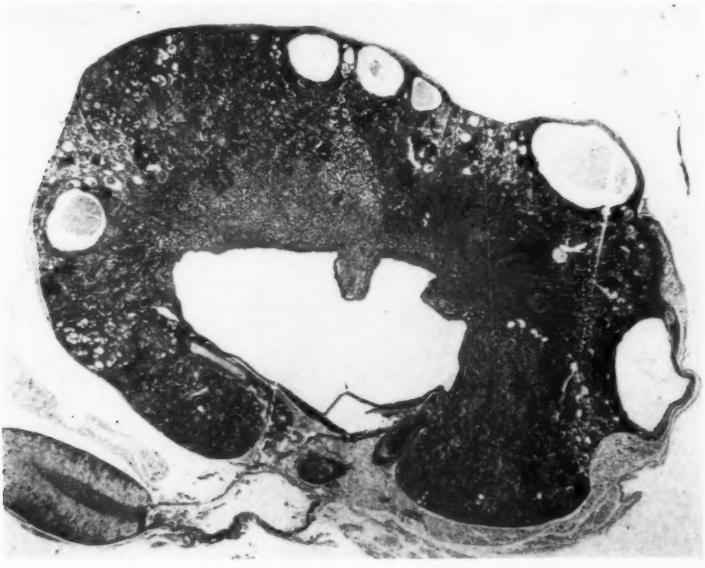

FIGURE 10.—Cystic stage of renal disease. × 12.

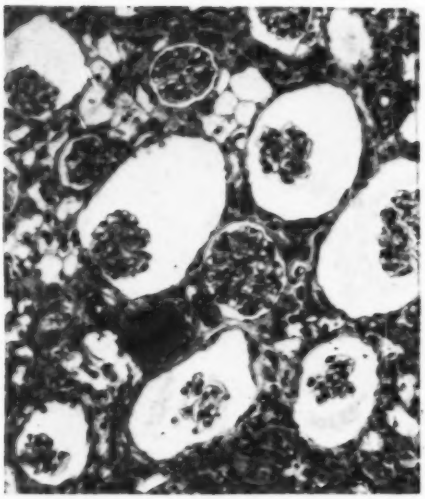

FIGURE 11.—Cysts arising from Bowman's space. × 150.

From the data in table 1, spontaneous amyloidosis and kidney disease appear to be infrequent in strain C3H mice. For additional data, the necropsy records of animals, which had been entered as amyloidosis or nephritis in the pathology-record file at the National Cancer Institute, were looked over, and many of the slides were examined. Observations from such material are naturally difficult to evaluate, but only three instances of associated amyloid and renal disease were noted in strain C3H animals that had not been subjected to experimental procedures. On the other hand, amyloid infiltration of the spleen and other organs was frequent in animals that had received repeated parenteral injections of a variety of substances, many of them carcinogenic. This amyloidosis was sometimes accompanied by a typical "strain A nephritis," but acute inflammatory conditions of the kidney and other histologic changes were also encountered in this unselected group of strain C3H animals. The youngest mice showing amyloidosis were 3 to 6 months old and had been inoculated with a rapidly growing sarcoma in which much necrosis and inflammatory exudate were present. Although no definite statements regarding material of this type can be made, it would appear that strain C3H mice can develop amyloidosis readily under certain experimental conditions and that such development may be followed by renal lesions. However, the spontaneous occurrence of the conditions is rare in this strain.

DISCUSSION

As yet no satisfactory name has been adopted for this kidney lesion. Twort and Twort (*5*) believed that most of the renal damage in mice is caused by infestation by *Klossiella muris* and hence applied the term "Klossiella nephritis" to a condition that appears to be similar to the disease found in strain A mice.

Infestation with Klossiella has been infrequent in our mice, and this parasite was never encountered among the mice used (table 1). Kidneys from 122 strain dba mice ranging from 6 to 14 months of age, which were available from another experiment in our files, were examined. Kidneys of 21 of these mice contained Klossiella organisms, but only 1 showed an interstitial nephritis and amyloid deposit in the glomeruli. The usual change was a generalized dilatation of the tubules, which were crowded with the parasite, and an enlargement rather than a contraction of the entire organ. The fact that this colony of animals has been kept separate from other stock animals may be the reason why the parasite has recently been found in this strain only.

It is thought that the implication of *Klossiella muris* as the causative agent in the development of nephritis and the associated "hyaline disease" (amyloid) was a natural conclusion on the part of Twort and Twort, which may be explained by the general infestation of their mice. However, since none of the parasites were found in our groups of mice, Klossiella nephritis is not an acceptable diagnosis.

Gorer (*6*) called the condition "cystic disease of Strain A mice." The cyst

formation is a later, and probably not an inevitable stage in development, hence a more inclusive term is desirable. Terms used in the records at the National Cancer Institute have been "nephritis," "glomerulonephritis," and "pyelonephritis." Nephritis is unsatisfactory because it includes other types of kidney damage; glomerulonephritis should not be applied to a condition where glomerular damage is at a minimum; and while pyelonephritis suggests the similarity of the microscopic picture to that seen in the human disease, this likewise is a misnomer since the involvement of the pelvis usually occurs in a local area after necrosis of the papilla. The nearest analogy to a kidney disease of man would probably be to amyloid contracted kidney, which is a relatively rare form of renal amyloidosis. For these reasons, the name "papillonephritis" is suggested, because it emphasizes the involvement of the papilla and does not suggest an analogy to any clearly differentiated type of nephritis observed in man.

The marked susceptibility of strain A mice and the apparent resistance of strain C3H to spontaneous amyloidosis are other instances where genetic factors appear to determine susceptibility to disease. Gorer emphasized the fact that it was strain A which most often developed cystic disease of the kidney, and he considered that a genetic factor was probably responsible. Since our observations indicate that amyloid infiltration of the papilla is responsible for the cyst formation in the kidney, it may be suggested that it is in the amyloid infiltration preceding the nephritis that genetic factors are to be sought.

The pathogenesis of this renal lesion, or papillonephritis, is thought to be as follows: In spontaneous amyloidosis, deposits in strain A mice tend to occur frequently in the interstitial tissue between the collecting tubules, which become partially or totally blocked; as a consequence, a histologic picture resembling pyelonephritis in man may result. Mallory et al. (*14*) have shown that partial urinary obstruction is necessary for the experimental production of pyelonephritis in rabbits. If similar conditions exist in mice, we may suppose that after the obstruction produced by amyloid infiltration in the papilla, resulting changes such as atrophy and dilatation of the tubules will take place in the cortex. It is not known whether secondary inflammatory reactions are necessary to the development of the final stage, but evidences of infection are not infrequent. In the final stage, numerous cysts may be present in the cortex.

SUMMARY

Spontaneous amyloidosis is of frequent occurrence in certain inbred strains of mice. In its distribution it bears a resemblance to primary or idiopathic amyloidosis of man, while experimentally produced amyloidosis in mice resembles the secondary disease of man.

Amyloid infiltration and a characteristic renal disease frequently occur together in certain inbred strains of mice.

Amyloidosis of other organs may occur without the renal change, but renal change in an animal in which amyloid cannot be found is extremely rare.

Amyloid deposits in the intertubular interstitial tissue of the papilla can be identified as the earliest lesion in the renal disease of strain A mice. This condition results in occlusion of the collecting tubules and produces the urinary obstruction which is an important factor in the development of a histologic change resembling pyelonephritis of the human kidney.

"Papillonephritis" is suggested as a name for this renal disease in mice, since it emphasizes the primary injury to the papilla and does not suggest a similarity to any common nephritis of man.

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ESTERASE ACTIVITY OF BLOOD SERUM OF FOUR STRAINS OF MICE

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Biochemical differences or alterations that are correlated with the occurrence of mammary tumors in mice are of obvious interest and importance as indications of changes in metabolism that may be related to carcinogenesis. Although the literature contains numerous reports describing such differences between strains of inbred mice, work on a larger number of strains has revealed no constant correlations of this kind. An investigation of the serum esterase of several strains of mice was stimulated by recent reports (*1*, *2*, *3*) that the butyric esterase activity of the blood serum of C57 Black mice, which have a low incidence of mammary tumors, is strikingly lower than of mice of strains C3H and A, which have a high incidence of mammary tumors, and that the values were raised in C57 Black animals and lowered in C3H mice that were reciprocally foster-nursed.

At least three factors are involved in the occurrence of mammary tumors in mice: (1) Genetic susceptibility; (2) the milk factor; and (3) hormonal stimulation. A strain of mice may have a low incidence of mammary tumors because the milk factor is lacking and yet be genetically susceptible to the formation of mammary tumors. Thus, biochemical and other differences may be related either to genetic susceptibility or to the presence or absence of the milk factor. Differentiation of these factors is necessary in order to evaluate the results.

EXPERIMENTAL PROCEDURE

Male and female mice of strains C3H, C57 Black, C, and I, and descendants of C mice that had been foster-nursed by C3H females were obtained from the colonies maintained at the National Cancer Institute. The mice varied in age between 3 and 14 months. They were maintained on Purina dog chow and an unlimited supply of water.

Blood was obtained from sectioned axillary and cervical blood vessels of unanesthetized animals. The serum was used within an hour. To 0.1 cc. of the serum were added 0.2 cc. of methyl butyrate and 5.0 cc. of phosphate buffer of pH 8.8. After incubation for 6 hours at 37.5° C., the system was titrated against 0.01 N potassium hydroxide with phenolphthalein as the indicator. The esterase activity of the blood serum was expressed in terms of cubic centimeters of 0.01 N potassium hydroxide required to neutralize the acid liberated by 0.1 cc. of the serum.

RESULTS

The results are presented in table 1. The esterase activity of the serum of C57 Black mice was significantly lower than the serum esterase of C3H females. Another low-mammary-tumor strain, I, however, had a higher serum esterase than did strain C3H. Furthermore, C mice, with an incidence of 1 percent of mammary tumors, showed the same esterase activity as did descendants of C mice that had been nursed by C3H females and that have continued to develop tumors of the breast in an incidence of over 70 percent.

TABLE 1.—*Esterase (butyric) activity of mouse serum*

Strain and description of mice	Sex	Incidence of mammary tumors [1]	Age of mice	Esterase determinations [2]	Esterase range	Esterase average
		Pct.	*Months*	*No.*		
C57 Black	F	0.5	3-4	10	5.8- 8.7	6.7
	M	<1	3-4	8	5.7- 8.1	6.9
C3H	F	91.4	3-5	12	8.1-14.8	11.4
	M	63.6	3-5	9	6.9- 8.7	7.9
C3H, with tumors	F	.....	6-12	8	4 -12.3	7.6
C	F	1.1	3-6	8	8.1-14.0	10.2
	M	0	4-9	10	8.2-14.1	10.9
C, fostered by C3H, F_{12}	F	76.8	4-6	7	7.3-13.0	10.2
	M	26.2	4-6	8	8.2-12.9	10.5
I	F	<1	12-14	5	12.4-16.1	14.4
	M	?	7-8	5	12.5-17.7	15.0

[1] In males, when implanted with diethylstilbestrol pellets.
[2] In many instances, the serum of 2 mice was pooled.

The results, in relation to genetic susceptibility and the presence or absence of the milk factor, are summarized in table 2.

TABLE 2.—*Esterase activity of serum of four strains of mice*

Mice	Genetic susceptibility	Milk factor	Incidence of mammary tumors	Serum esterase
C57 Black	Low	Absent	<1	6.8
I	do	do	<1	14.8
C	High	do	1	10.5
C fostered	do	Present	75	10.3
C3H	do	do	90	11.4

The data indicate that in these mice the presence or absence of the milk factor, or the degree of susceptibility was not correlated with the esterase activity of the blood serum.

There are several minor differences in the technique employed by Khanolkar and Chitre (*2*) and in our experiment. Most of our animals were younger than 6 months, whereas theirs were older. In neither their data nor ours, however, was there evidence that the serum esterase was affected by the age of the animals. We obtained blood from unanesthetized animals, whereas Khanolkar and Chitre bled mice from the portal vein after killing them with chloroform. The possible effect of difference in diet is unknown; Khanolkar and Chitre (*3*) suggest that a change from fresh whole milk to meat and vegetable oils in the diet reduced the liver esterase in their animals. In the matter of esterase determinations, Khanolkar and Chitre state that "a few drops" of ethyl butyrate were used (*2*). We employed 0.2 cc. of methyl butyrate, since ethyl butyrate is less soluble. There is no obvious reason to suppose that these deviations in technique altered the essential results.

The esterase activity of the serum of C3H males was lower than that of C3H females, only two of nine values for the males being at the lowest range of the values for the females. The significance of these results is not established. Of nine C3H females with mammary tumors, six had esterase values below the range for normal females. A decrease in serum esterase activity in tumor-bearing animals has been reported (*4*). The mice were in good condition, but the tumors, although not externally ulcerated, were large, measuring up to 20 by 20 mm. in diameter.

The level of blood-serum esterase activity in four strains of mice was not correlated with the incidence of mammary tumors in the animals.

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ESTERASE (BUTYRIC ESTERASE) ACTIVITY OF NORMAL AND NEOPLASTIC TISSUES OF THE MOUSE

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Among the enzyme systems in normal and in neoplastic tissues investigated in the series of studies from this laboratory, there have been systems which catalyzed transformations of amino acids (*1*, *2*), of carbohydrate (*3*), of phosphate esters (*4*, *5*), of hydrogen peroxide (*5*, *6*), of protein (*7*), of purine bases (*3*, *4*), of nucleic acids (*5*, *6*), and of cytochrome (*8*). An important class of substrate not investigated thus far has been the fats. A study has therefore been made of the esterase activity of a number of normal and neoplastic tissues of the mouse, using methyl butyrate as the substrate.

EXPERIMENTAL PROCEDURE

The esterase activity of the various tissues was determined upon extracts of the tissues prepared by grinding weighed amounts of tissues removed from the freshly killed mice with three times the amount of distilled water in the presence of washed sand.[1] The suspensions were allowed to stand for 18 hours in the ice box and were then centrifuged for a few minutes at 1,500 r. p. m. The clear supernatant fluid served as the source of the esterase. One cubic centimeter of the extract was thus roughly equivalent to 300 mg. of wet tissues.

[1] The tumors were donated for this study by the members of the staff of this Institute: hepatomas 7A/77 and 587 in A mice by Dr. Eschenbrenner; hepatoma A in C3H mice, the spontaneous mammary tumor in C3H mice, and the intestinal adenocarcinoma in A mice by Dr. Thompson; lung tumor F in A mice and S37 and CR180 in dba mice by Dr. Andervont; the melanomas in dba and in C mice by Dr. Algire; the primary methylcholanthrene-induced tumor in LAF mice by Dr. Shear; the salivary-gland tumor in C mice by Dr. Dalton; and the squamous-cell carcinoma in A mice, the spindle-cell sarcoma in C3H mice, and the lymphoma 163 in C3H mice by Dr. Dunn.

The determinations were made as follows: One cubic centimeter of the extract was treated with 1 cc. of phosphate buffer at pH 7.2 and 0.5 cc. of methyl-n-butyrate. Simultaneously, similar digests were prepared containing tissue extract diluted successively by half (with distilled water), by one-quarter, by one-eighth, etc. The series of dilutions were employed for the reasons stated in earlier papers (*1*, *4*), namely, because the maximum effect of the enzyme elicited in any one of the tissues was not known beforehand. The digests were allowed to incubate for 2 hours at 37° C. Controls of the tissue extracts alone and of the substrate alone were run simultaneously.

At the end of the 2-hour incubation period, 5 cc. of 95 percent alcohol was added to each digest with shaking. The tubes containing the alcoholic mixture were centrifuged for a few minutes at 1,500 r. p. m., and the supernatant fluid was separated. A drop of 1 percent phenolphthalein was added to the supernatant of each tube and the mixture titrated with 0.1 N alcholic potassium hydroxide (90 percent alcohol). The alkali neutralized the butyric acid formed from the hydrolysis of the methyl butyrate. Duplicate determinations agreed within 10 percent.

Normal tissues were obtained from strain C3H, A, and dba mice, as well as from a hybrid Swiss group. No strain differences were apparent. All the tumors were transplants, with the exception of the primary tumor induced by administration of methylcholanthrene.

The data are given in table 1.

TABLE 1.—*Esterase activity of normal and neoplastic mouse tissues* [1]

Tissue	Tissue weight in extract [2]	0.1 N alcoholic potassium hydroxide consumed [3]	Q [4]
	Milligrams per cubic centimeter	*Cubic centimeters*	x 10 [4]
Normal tissues: [5]			
Heart	300	0.4	13
	150	.2	13
	75	.1	13
	38	0	.....
Average			13
Skeletal muscle	300	.4	13
	150	.2	13
	75	.1	13
	38	0	.....
Average			13
Spleen	300	3.1	103
	150	1.5	100
	75	.9	120
	38	.4	105
	19	.2	105
	9	.1	111
Average			108
Brain	300	.2	7
	150	.1	7
	75	0	.....
	38	0	.....
Average			7
Liver	300	4.3	.....
	150	3.6	.....
	75	2.9	386
	38	1.5	394
	19	.8	421
	9	.4	444
Average			411
Kidney	300	3.0	100
	150	1.6	106
	75	.9	120
	38	.4	105
	19	.2	105
	9	.1	111
Average			108
Lung	300	1.9	63
	150	1.0	66
	75	.5	66
	38	.3	78
Average			68
Intestinal mucosa	300	4.3	.....
	150	4.1	.....
	75	3.9	.....
	38	3.7	973
	19	1.8	948
	9	.9	1,000
Average			973
Pancreas	300	6.6	.....
	150	6.6	.....
	75	5.0	.....
	38	3.9	.....
	19	3.2	1,684
	9	1.6	1,777
	4	.8	2,000
Average			1,820

TABLE 1.—*Esterase activity of normal and neoplastic mouse tissues* [1]—Continued

Tissue	Tissue weight in extract [2]	0.1 N alcoholic potassium hydroxide consumed [3]	Q [4]
	Milligrams per cubic centimeter	*Cubic centimeters*	x 10 [4]
Normal tissues [5]—Con.			
Gastric mucosa	300	1.3	43
	150	.7	46
	75	.4	53
	38	.2	52
Average			48
Lymph nodes	300	.7	23
	150	.4	26
	75	.2	27
	38	0	.....
Average			25
Thymus	300	.1	3
	150	0	.....
	75	0	.....
	38	0	.....
Skin	300	.1	3
	150	0	.....
	75	0	.....
	38	0	.....
Neoplastic:			
Hepatoma 7A/77 [6]	300	3.6	.....
	150	3.0	200
	75	1.6	212
	38	.8	210
Average			207
Hepatoma 587 [7]	300	3.0	100
	150	1.5	100
	75	.8	106
	38	.4	105
Average			103
Hepatoma A [8]	300	3.2	.....
	150	.....	.....
	75	1.2	160
	38	.7	184
Average			172
Melanotic melanoma	300	.9	30
	150	.4	26
	75	.2	26
	38	0	.....
Average			27
Amelanotic melanoma	300	1.0	33
	150	.4	26
	75	.2	26
	38	.1	26
Average			27
Spontaneous mammary carcinoma	300	.8	27
	150	.4	26
	75	.2	26
	38	.1	26
Average			26
Lung tumor F	300	.2	6
	150	0	.....
	75	0	.....
	38	0	.....

See footnotes at end of table.

TABLE 1.—*Esterase activity of normal and neoplastic mouse tissues* [1]—Continued

Tissue	Tissue weight in extract [2]	0.1 N alcoholic potassium hydroxide consumed [3]	Q [4]
	Milligrams per cubic centimeter	*Cubic centimeters*	x 10 [4]
Neoplastic—Continued. Salivary gland tumor	300	0 4	13
	150	.2	13
	75	0	.....
	38	0	.....
Average			13
Primary methylcholanthrene-induced sarcoma.	300	.2	6
	150	.1	6
	75	0	.....
	38	0	.....
Average			6
S37	300	.2	6
	150	0	.....
	75	0	.....
	38	0	.....
CR180	300	.2	6
	150	0	.....
	75	0	.....
	38	0	.....
Squamous cell carcinoma of stomach.	300	.7	23
	150	.3	20
	75	.1	14
	38	0	.....
Average			19
Intestinal adenocarcinoma.	300	.3	10
	150	.2	12
	75	0	.....
	38	0	.....
Average			11
Spindle-cell sarcoma	300	.2	6
	150	0	.....
	75	0	.....
	38	0	.....
Lymphoma 163	300	.3	10
	150	.1	7
	75	0	.....
	38	0	.....
Average			8

[1] Incubation mixtures made up of 1 cc. extract+1 cc. phosphate buffer at pH 7.2+0.5 cc. methyl-n-butyrate. Time of incubation, 2 hours.
[2] Tissue weight refers to wet weight.
[3] Corrected for blanks.
[4] Q=Corrected cubic centimeters 0.1 alcoholic potassium hydroxide consumed in 2 hours / Milligrams per cubic centimeter
[5] From C3H, dba, and Swiss mice. Tumors are from strains of mice listed in footnote 1 of the text.
[6] Primary tumor had been induced by injection of carbon tetrachloride.
[7] Primary tumor had been spontaneous.
[8] Primary tumor had been induced by injection of 2-amino-5-azotoluene.

At the higher dilutions of the more active tissue extracts and at the lower dilutions of the less active tissue extracts there appears to be a linear relation between dilution and alkali consumed. It is possible to formulate this relation as follows:

$$Q=\frac{\text{Corrected cubic centimeters 0.1 N alkoholic potassium hydroxide consumed in 2 hours}}{\text{Milligrams per cubic centimeter tissue etract}} \quad (1)$$

The average values of Q are given in table 1, and it is clear that a fairly consistent group of values is given for each tissue within certain limits of concentration. The failure of the relation to hold for the more active tissues at high concentrations is very likely to be attributed to the inactivation of the enzyme by the acid formed from the ester hydrolysis. At low extract concentrations of the less active tissues, the acid formed was so slight, the experimental error consequently so high, that no attempt could be made to apply the above relation.

Equation 1 is similar to that observed to hold for the action of phosphatase in various normal and neoplastic tissues (*4*).

DISCUSSION

The order of decreasing esterase activity per milligram of tissue in the various normal mouse tissues was as follows: pancreas, intestinal mucosa, liver, kidney or spleen, lung, gastric mucosa, lymph nodes, heart or skeletal muscle, brain, and thymus or skin. The activity, in terms of Q (table 1) ranged from 1,820 for pancreas to 3 for thymus or skin.

With one exception, the mouse tumors had a rather low order of esterase activity. The exception concerned the transplanted hepatomas (table 1). The activity among the group of tumors studied ranged from a Q value of 207 for hepatoma 7A/77 to a Q value of 6 for several of the tumors. The range among the normal tissues was, as often observed before (*5*), considerably greater than among a group of tumors of the same species of animal.

Comparison of the homologous tissues is of some interest. Although the Q values of the hepatomas were all lower than that of normal liver, the drop in esterase activity when the mouse liver

cell became neoplastic was not so great as in the case of the intestinal mucosa. The Q value of the latter tissue was 973, that of the intestinal adenocarcinoma 11 (table 1). The change of the mucosa into the adenocarcinoma was accompanied by a greater change in the esterase content than that observed in the change of the liver cells into the hepatoma. The normal lymph nodes possessed a relatively low esterase activity; when these nodes became neoplastic, there was a decrease to about a third in the activity of this enzyme.

The etiology of the three hepatomas described in table 1 was different. The esterase activity was, however, of the same order of magnitude for all three tumors.

SUMMARY

The esterase activity of 13 normal and of 15 neoplastic tissues of the mouse was determined at various dilutions of the tissue extracts. At higher dilutions of the active tissues and lower dilutions of the less active tissues, a linear relation between activity and concentration of tissue was apparent. The range in activity among the normal tissues studied was far greater than among the tumors. The activity of the hepatomas and intestinal adenocarcinoma was considerably less than that of the respective normal tissues of origin of these tumors, namely, liver and intestinal mucosa, but the difference between liver and hepatoma was less than the difference between intestinal mucosa and the adenocarcinoma.

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COMPARATIVE OXIDASE ACTIVITY OF MELANOTIC AND AMELANOTIC MELANOMAS[1]

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It has long been known that melanomas may or may not contain the melanin pigment. The formation of the pigment in the melanotic Harding-Passey tumor in mice has been shown to be due in part to the presence of the enzymes tyrosinase and dopa oxidase (*1*). Suspensions of this tumor catalytically oxidize tyrosine and dihydroxyphenylalanine (dopa) to melanin with the uptake of five and of four atoms of oxygen, respectively. Are the amelanotic tumors lacking in the pigment because these enzymes are absent or are the latter present and nonfunctioning because of some lack of availability of the substrates? The attempt to find an answer to these questions forms the basis of the present investigation.

EXPERIMENTAL PROCEDURE

For the best comparison between melanotic and amelanotic tumors, it was desirable to conduct the studies on material arising from a single source. For this purpose, therefore, the pigmented and the nonpigmented portions of the transplanted mouse melanoma S91 were employed. The characteristics of this tumor will be described in the paper by Algire. It needs to be stated here only that the amelanotic portions of the transplanted tumor appear on the periphery of the latter and must be carefully dissected from the melanotic portion. The amelanotic portion is not completely free of pigment, its color is a grayish white, but the difference between this and the black-colored melanotic portion is sufficiently striking so that comparisons may be made with confidence. C and dba strains of mice were used, but no differences in the results obtained were observed between the two strains. For further comparison, the transplanted Harding-Passey pigmented melanoma in C strain mice was studied; little if any difference in the results obtained was observed between this tumor and the pigmented S91 melanoma.

The animals were killed by neck dislocation and the tumors removed immediately. The tissues were ground with sand in a glass mortar and homogenized with four times the amount of phosphate buffer. This suspension, poured from the sand, formed the source of the tyrosinase and dopa oxidase enzymes investigated. Each cubic centimeter of the suspension contained roughly 250 mg. of tissue.

Tyrosinase and dopa oxidase activities were measured by adding to a Warburg flask 1 cc. of the tissue suspension homogenized in phosphate buffer at pH 6.5 and 2 cc. of the buffer at this pH. In the side arm of the flask was placed 0.5 cc. of an aqueous solution of tyrosine (0.01M) or of dihydroxyphenylalanine (0.01M.). Appropriate blanks were likewise run on the suspension alone, and on each of the substrates. These gave a negligible uptake of oxygen. The effect of KCN (0.01 M) on the activity of tyrosinase and of dopa oxidase was observed by substituting 1 cc. of the cyanide solution for 1 cc. of the buffer. The flasks after attachment to the single manometers were shaken in the bath at 38° C. for 15 minutes, the flasks were tipped and the manometers closed, and the oxygen uptake was measured every 5 minutes over a 2-hour period.

The activity of cytochrome oxidase was measured on tumor suspensions equivalent to 100 mg. of tissue per cubic centimeter of phosphate buffer at pH 7.2. The mixtures were made by adding 1 cc. of the suspension to 1 cc. of buffer at pH 7.2 and 1 cc. of cytochrome *c* solution (1×10^{-4}M); in the side arm of the flask was placed 0.3 cc. of *p*-

[1] The growth and pathology of the melanoma will be described by Algire in a subsequent issue of the Journal.

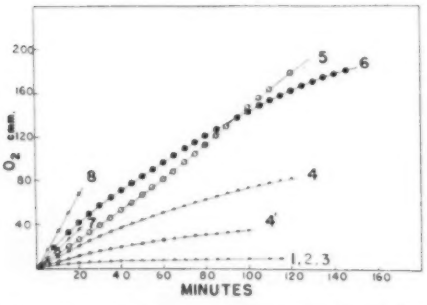

FIGURE 1.—Curves 1, 2, 3, Data on the amelanotic melanoma S91 suspensions treated respectively with tyrosine (pH 6.5), dihydroxyphenylalanine (pH 6.5), and *p*-phenylenediamine (pH 7.2); curve 4, Data on the melanotic melanoma suspensions (S91 or Harding-Passey) in buffer and treated with *p*-phenylenediamine (pH 7.2); curve 4', same as curve 4, except that 1 cc. of 0.01 M KCN was added in place of 1 cc. buffer; curve 5, Data on the melanotic melanoma suspensions (S91 and Harding-Passey) in buffer treated with tyrosine (pH 6.5); curve 6, data on the melanotic melanoma suspensions (S91 and Harding-Passey) in buffer with dihydroxyphenylalanine (pH 6.5); curve 7, data on the amelanotic melanoma suspensions S91 treated with *p*-phenylenediamine and excess cytochrome *c* (pH 7.2); curve 8, data on the melanotic melanoma suspensions (S91 and Harding-Passey, treated with *p*-phenylenediamine and excess cytochrome *c* (pH 7.2).

Note: No observable uptake of oxygen by the tyrosine, dihydroxyphenylalanine, or *p*-phenylenediamine, or by any of the tissue suspensions alone in buffer or in KCN, or by the tissues treated with tyrosine and dihydroxyphenylalanine in the presence of KCN, or by the amelanotic melanoma in the presence of *p*-phenylenediamine and KCN.

phenylenediamine (0.2 M). In order to calculate the activity of the cytochrome oxidase, the effect of the autoxidizable components in the tissue was subtracted from the total oxygen uptake; this was accomplished by substituting 1 cc. of KCN solution (0.01 M) for the 1 cc. of cytochrome *c*. The cytochrome oxidase activity is thus given by the expression:

$$Qc = \frac{\text{Cubic millimeters } O_2 \text{ (with cytochrome c)} - \text{Cubic millimeters } O_2 \text{ (with KCN)}}{\text{Milligrams wet tissue} \times \text{hours}}$$

After mixing the contents of the flasks with that of the side arm, the uptake of oxygen was measured every 5 minutes for a 20-minute period. During the period the oxygen uptake was linear with time.

The mixture of the melanotic tumor suspensions with KCN and *p*-phenylenediamine gave the rather surprising result of a significant absorption of oxygen, amounting roughly to 20 percent of the absorption when the KCN was replaced by cytochrome *c*. It was considered desirable to extend this observation further, and consequently longer periods of incubation were studied of mixtures of the tumor suspensions in the presence of *p*-phenylenediamine both with and without the presence of KCN. These mixtures were composed throughout of 1 cc. of the suspension in buffer of pH 7.2, plus 1 cc. of buffer at this pH, plus either 1 cc. of buffer or 1 cc. of KCN solution (0.01 M); in the side arm was 0.3 cc. of *p*-phenylenediamine solution (0.2 M). The temperature throughout was 38° C.

All determinations described herein were run in duplicate on several batches of each kind of tumor. The results of the duplicate determinations were invariably within 5 percent of each other; the results of different batches of the same tumor agreed with each other within the range of ±10 percent. The representative data are graphically described in figure 1.

RESULTS

TYROSINASE AND DOPA OXIDASE

The curves in figure 1 reveal that the pigmented or melanotic melanomas, whether the Harding-Passey or the S91 tumor, yield active preparations of the two enzymes, tyrosinase and dopa oxidase (curves 5 and 6). The amount of dihydroxyphenylalanine used per flask would require about 220 mm.^{3} of oxygen for its oxidation if four atoms of oxygen were utilized for the conversion of this substrate to melanin (*1*). Curve 6 shows that this value is indeed approached. The period of observation was not sufficiently long to determine the maximum uptake of oxygen by the mixture treated with tyrosine (which would require

about 300 mm.^{3} or five atoms), but it is obvious that the absorption by this substrate exceeded that of the dihydroxyphenylalanine (curve 5). At the end of the observation period, the control solutions of the suspensions alone or with the substrate in the presence of KCN were brown and had absorbed little or no oxygen, whereas the mixture with either substrate which had absorbed considerable oxygen were coal-black from the formed melanin. The evidence of the amount of oxygen absorbed and of the melanin formed preclude the possibility that amino acid oxidase may have been responsible for the effects observed. The data obtained are in agreement with those of Hogeboom and Adams (*1*) on the pigmented Harding-Passey melanoma.

In striking contrast with the results obtained with the pigmented melanomas, the suspension of the nonpigmented melanoma revealed little activity in the presence of tyrosine or of dihydroxyphenylalanine (curves 1 and 2). The very small amount of activity shown may have been due in part to the presence of the few pigmented cells in the amelanotic portions of the tumor from which the suspensions were prepared. In any event, the amelanotic portion of the melanoma was acutely deficient at least in the two enzymes studied, as further shown by the very light color of the mixtures at the end of the incubation period of over 2 hours.

ABSORPTION OF OXYGEN IN THE PRESENCE OF *p*-PHENYLENEDIAMINE

The addition of *p*-phenylenediamine to the suspension of either of the pigmented melanomas resulted in an appreciable uptake of oxygen by the mixture (curve 4). The absorption by the latter is only partially sensitive to cyanide, a considerable proportion, roughly 50 percent of the uptake in the absence of cyanide, remaining effective (curve 4′). The nature of the cyanide-insensitive enzymatic system or systems under these conditions is not known at the present time. Examination of the suspension in the presence of reducing agents with a hand spectroscope revealed no bands characteristic of cytochrome *b*.

Again, in the mixtures with suspensions of the amelanotic melanoma S91, the addition of *p*-phenylenediamine produced very little absorption of oxygen. The small amount of absorption observed may have been due in part to the few melanotic cells present (curve 3).

CYTOCHROME OXIDASE

The uptake of oxygen by the suspension of the melanotic melanomas in the presence of *p*-phenylenediamine and of excess cytochrome *c* amounts in various preparations from 1.7 to 1.9 mm.^{3} per milligram wet weight per hour (curve 8). When 0.01 M KCN is substituted for cytochrome *c*, the uptake of oxygen by the mixture (for the first 20 minutes) amounts to about 0.4 mm.^{3} per milligram wet weight per hour (curve 4′). The difference amounts to 1.3 to 1.5 mm.^{3} per milligram per hour and is a measure of the cytochrome oxidase activity of the suspensions.

The uptake of oxygen by the suspension of the amelanotic melanoma in the presence of *p*-phenylenediamine and of excess cytochrome *c* amounts in various preparations from 1.1 to 1.4 mm.^{3} per milligram wet weight of tissue per hour (curve 7). When 0.01 M KCN is substituted for the cytochrome *c*, the uptake of oxygen is negligible.

It would appear that the cytochrome oxidase activity of the melanotic melanoma was slightly greater than that of the amelanotic tumor, but it is not believed that this difference is particularly significant or important.

DISCUSSION

The melanotic melanomas are characterized by the possession of (1) cyanide-sensitive tyrosinase, (2) cyanide-sensitive dopa oxidase, and (as shown in this paper) (3) enzymatic system or systems which oxidize *p*-phenylenediamine and which are only partially cyanide-sensitive. It has been claimed that dopa oxidase exists in the normal melanoblast (*2*, *3*), but the evidence, as

in comparable efforts to observe a skin tyrosinase (see (*1*)), has been inconclusive; the dilution of the enzymes if they were present precluded in any event a definite estimation. Many normal tissues such as the heart, kidney, liver, and brain, and some animal tumors, when in suspension, absorb oxygen in the presence of *p*-phenylenediamine, the normal group absorbing more per milligram than the tumor group. In either case the oxygen uptake is completely abolished by addition of cyanide.[2] The uptake of oxygen by these tissues under these conditions was most probably due to the cytochrome oxidase-cytochrome *c* system. The distinguishing characteristic of the melanotic melanomas lies therefore not so much in the presence of a highly active *p*-phenylenediamine oxidizing system, which some normal tissues and some tumors possess in equal or less degree, but in the fact that a considerable proportion (roughly 50 percent) of this system is insensitive to addition of 0.01 M cyanide.[3]

The amelanotic melanoma studied was nearly lacking in tyrosinase and dopa oxidase and in the *p*-phenylenediamine-oxidizing system. The residual activity of these systems in the tumor was completely abolished by cyanide. The amelanotic melanoma behaved very much like all other nonpigmented tumors. This melanoma evidently does not produce melanin in the presence of tyrosine or of dihydroxyphenylalanine because it lacks the necessary enzymes, and not because of some possible unavailability of the substrates to the tissue. The mechanism for melanin production is apparently independent of the malignant nature of the melanoma. As far as the cancer problem is concerned, the melanin produced may be of only academic curiosity.

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[3] Recent study of a deeply pigmented human melanoma gave results essentially similar to those obtained with melanotic mouse melanomas except that the activity of the enzymatic systems in the human tumor was far greater than that in the mouse tumors [4]. The suspensions of the human tumor possessed not only a very high *p*-phenylenediamine-oxidizing capacity but close to 100 percent of this activity was insensitive to cyanide; this tumor also possessed highly active tyrosinase and dopa oxidase systems. An amelanotic human tumor studied at the same time revealed little if any activity in the presence of *p*-phenylenediamine.[5] The cytochrome oxidase activity of the melanotic and amelanotic human melanomas was nearly the same. The results on the mouse and on the human melanomas are thus essentially similar.

[4] [5] See footnote 2.

SUMMARY

The activity of tyrosinase and of dihydroxyphenylalanine oxidase (dopa) in the amelanotic and melanotic portions of the transplanted melanoma S91 in C and dba mice was investigated by following the rate and amount of oxygen absorption in mixtures of the tissue suspensions with each of the substrates. The melanotic portions of this tumor, as well as the melanotic Harding-Passey melanoma, revealed active cyanide-sensitive systems. The amelanotic portions of the S91 melanoma revealed very little activity of these systems.

The melanotic tumors, in contrast with the amelanotic, showed considerable oxygen uptake in the presence of *p*-phenylenediamine. The small amount of oxygen absorption shown by the amelanotic melanoma was completely abolished by cyanide. Of the larger amount of oxygen absorption shown by the melanotic melanomas in the presence of *p*-phenylenediamine, about one-half was insensitive to the addition of cyanide.

It would appear, therefore, that the melanotic melanomas are distinguished by the possession not only of tyrosine and of dopa oxidase but also of a cyanide-insensitive system, which oxidizes *p*-phenylenediamine. The amelanotic melanoma was nearly completely lacking in all these systems.

The activity of cytochrome oxidase in the melanotic and in the amelanotic melanomas was observed to be of the same order of magnitude.

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NOTE ON THE CYSTINE OXIDASE ACTIVITY IN NORMAL AND NEOPLASTIC TISSUES OF THE MOUSE

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Cystine oxidase catalyzes the degradation of cystine or of cysteine into pyruvic acid, hydrogen sulfide, and ammonia (*1*). Cystine peptides are also affected by the presence of this enzyme (*2*). The catalytic properties of the enzyme are equally effective under aerobic and anaerobic conditions (*1*, *2*) and may be exhibited in cell-free extracts of certain tissues. It is distinct from amino acid oxidase because of (1) its equal effectiveness in the presence and absence of oxygen, (2) its high activity in mouse liver, which shows no appreciable amino acid oxidase activity, (3) its greater activity in rat liver than in rat kidney, (4) its far greater action on the degradation of cystine than of alanine, and (5) its capacity to affect cystine in peptide form.

In contrast with normal liver, hepatomas contain no demonstrable cystine oxidase (*2*). Other mouse tumors and normal tissues were therefore investigated for the presence or absence of this enzyme.

The procedure used previously for the investigation of this enzyme in liver and in hepatomas (*2*) was varied in a few details. The time of incubation was extended from 2 to 4 hours, and the amount of extract used was halved. The method finally used was as follows: Tissues were removed from the freshly killed mice and ground with sand and with three times the weight of water. The suspensions were allowed to stand overnight in the ice box and were centrifuged at 1,500 r. p. m. the following morning. The supernatant liquid served as the source of the enzyme. One cubic centimeter of the extract was thus roughly equivalent to 300 mg. of wet tissue. One cubic centimeter of the extract was treated with 1 cc. of phosphate buffer at pH 7.2 and 1 cc. of *l*-cystine solution (0.025 M in 0.1 N sodium chloride).[1] The mixtures were incubated at 38° C. for 4 hours, then treated with a drop of 1 percent acetic acid, set in boiling water for 10 minutes, and cooled. The mixtures were then treated with distilled water, saturated potassium carbonate solution, and a drop of caprylic alcohol and aerated for ammonia as before (*2*). The amount of ammonia found, less that contributed by the extract itself, is a function of the cystine oxidase activity of the tissue. Duplicate determinations agreed within 10 percent. With one exception, all the tumors studied were transplants.[2] Normal tissues were obtained from C3H, dba, and Swiss mice. No strain differences were observed. The collected data are given in table 1.

The data (table 1) show that of all the normal tissues studied, only liver, kidney, and pancreas, in descending order of activity, possess cystine oxidase activity. All the other normal tissues and all of the tumors studied possess no activity which could be measured under the conditions employed. Cystine oxidase is, therefore, the first sys-

[1] Solutions of *l*-cystine were prepared by dissolving the amino acid in 0.1 N sodium hydroxide followed by neutralization with 1.0 N hydrochloric acid. Appropriate volumes of such solutions were rapidly pipetted into the digestion flasks before crystallization of the cystine began. Fresh solutions were prepared before each set of determinations.

[2] The source of the tumors is given in the accompanying paper on esterase (*3*).

TABLE 1.—*Cystine oxidase activity of normal and neoplastic mouse tissues* [1]

Tissues	Ammonia N produced		
	Extract+substrate (Mols x 10^{-6})	Extract alone (Mols x 10^{-6})	Difference [2] (Mols x 10^{-6})
Normal:			
Liver	9.0	2.8	6.2
Kidney	5.2	2.6	2.6
Pancreas	3.6	2.0	1.6
Lung	1.1	1.2	(−.1)
Brain	1.3	1.2	(.1)
Gastric mucosa	1.1	1.0	(.1)
Intestinal mucosa	3.1	3.1	0
Lymph nodes	.2	.2	0
Spleen	1.1	1.0	(.1)
Thymus	.4	.4	0
Skeletal muscle	1.1	1.1	0
Heart muscle	1.1	1.1	0
Skin	.3	.3	0
Neoplastic:			
Hepatoma 7A/77	2.8	2.9	(−.1)
Hepatoma A	2.8	2.7	(.1)
Hepatoma 587	3.0	3.0	0
Spontaneous mammary carcinoma	2.2	2.2	0
Salivary gland tumor	1.2	1.1	(.1)
Lung tumor F	1.4	1.4	0
Melanotic melanoma	.9	.8	(.1)
Amelanotic melanoma	1.5	1.4	(.1)
Squamous-cell carcinoma of the stomach	1.1	1.2	(−.1)
S37	.8	.8	0
CR180	.8	.8	0
Intestinal adenocarcinoma	1.9	1.8	(.1)
Primary sarcoma induced with methylcholanthrene	1.4	1.4	0
Spindle-cell sarcoma	.8	.7	(.1)
Lymphoma 163	1.0	1.0	0

[1] Determined by ammonia formed in the degradation of *l*-cystine as substrate. Test run consists of 1 cc. extract (equivalent to 300 mg. wet weight of tissue)+1 cc. phosphate buffer at pH 7.2+1 cc. *l*-cystine solution (0.025 M in 0.1 N sodium chloride). Control was the same as the test, except that 1 cc. water was substituted for cystine solution. The cystine solution alone yielded no ammonia under the conditions employed.

[2] Results within experimental error are enclosed in parentheses.

tem observed which, although present in several normal tissues, is completely absent in all tumors studied. All enzymes hitherto studied (*3*, *4*, *5*) have been present in all tumors to greater or less degree. The absence of cystine oxidase from a large number of normal tissues, however, renders it difficult to ascribe its absence from tumors to an exclusive property of neoplasms. Nevertheless, the fact that hepatomas in both rats and mice contain no demonstrable cystine oxidase in contrast with the highly active liver, suggests, in conjunction with the other data in table 1, that tumors in general can exist in the absence of cystine oxidase.

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EFFECT OF A LOW LYSINE DIET ON MAMMARY-TUMOR FORMATION IN STRAIN C3H MICE

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The Andervont subline of strain C3H virgin female mice when fed Purina dog chow had a mammary-tumor incidence of 97.4 percent (*1*). However, if these mice were maintained from the time of weaning on a diet low in cystine, no mammary tumors developed (*2*). The implantation of stilbestrol pellets subcutaneously in mice ingesting the low cystine diet raised the incidence of such tumors from zero to 45 percent (*3*), showing that the suppression of mammary-gland-tumor development was due, in part at least, to a lack of estrogenic hormone.

It is known that the mammary gland grows under the influence of estrus. Turner and Gomez (*4*) described the elongation of the end buds during estrus and their partial regression near the next proestrous stage. It is by this means that the extent and complexity of the duct system of the mammary gland was increased until full virginal development was attained. Because of the undeveloped state of the mammary glands of virgin female mice fed the low cystine diet, as was shown by mounts of the mammary tissue prepared at necropsy (*5*), it seemed probable that mice as well as rats (*6*) did not show estrus on a low cystine diet.

A diet low in lysine also has been shown to have an effect on estrus in the rat. Evans and Bishop (*7*) fed 13 young rats a diet in which the protein was supplied by 60 percent wheat. Seven of the rats showed no estrus after the diet was started, and only 3 had more than 1 cycle. Courrier and Raynaud (*8*) found that when rats ingested an 18-percent gliadin diet, estrus stopped. Pearson (*6*) fed 65-day-old rats an 18-percent gliadin diet and found that after 2 or 3 normal cycles estrus usually ceased, although in a few cases irregular or prolonged cycles occurred. When the level of gliadin was 10 percent (*9*), estrus ceased entirely.

Thus it seems that in the rat a diet low in lysine tends to suppress estrus. If the same influence is exerted in a mouse, then interference with estrus should result in an altered mammary-tumor incidence.

EXPERIMENTAL PROCEDURE

Strain C3H virgin female mice, supplied by Dr. H. B. Andervont, of this Institute, were fed from weaning a diet low in lysine:

	Percent
Gliadin (Huron Milling Co., Harbor Beach, Mich.)	18
Cornstarch	50
Salts (Hubbell, Mendel, and Wakeman)	4
Crisco	20
Cod-liver oil	3
Ryzamin B No. 2 (Burroughs-Wellcome)	5
Total	100

On this diet, weanling strain C3H mice were able to gain weight only very slowly (about 1 gm. in 2 months). The average weight of the female mouse of this strain at weaning is 16 to 18 gm. At 9 months of age the surviving mice fed the low lysine diet weighed an average of 21 gm. The mice were kept two in a cage, and weekly records of body weights were made. At each weighing the mice were examined for mammary-gland tumors. When a tumor was observed, a note of it was made in the record, and the mouse allowed to live for a week or two longer. All animals were necropsied. Sections of mammary-gland tumors were prepared, and the diagnosis was made by Dr. A. B. Eschenbrenner, of this Institute.

RESULTS AND DISCUSSION

The results are summarized in table 1. The mice fed the low lysine diet showed a 25-percent incidence of mammary tumors at an average age of 12.9 months. While the mice on the low cystine diet developed no tumors at all, it was probable from the appearance of mammary-tissue mounts and from some observations of vaginal smears that

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TABLE 1.—*Appearance of spontaneous mammary tumors or of death in strain C3H virgin female mice*

[Numerator=number of mice developing tumors]
[Denominator=number of mice dying free of tumor]

Diet	Number of mice	Mice developing tumors or dying free of tumors according to age (in months)																Total	Tumor incidence	Average age
		5	6	7	8	9	10	11	12	13	14	15	16	17	18	22	28			
																			Pct.	*Months*
Low lysine	32	0/0	0/2	0/0	0/1	2/1	1/0	0/0	2/5	0/0	0/2	1/2	0/1	1/1	0/1	1/4	0/4	8/24	25.0	12.9
Chow [1]	350	3/0	11/1	20/0	35/0	63/0	51/0	56/1	41/1	26/1	13/1	10/2	5/0	6/0	1/1	0/1	....	341/9	97.6	10.4

[1] Data from Andervont (*1*).

estrous cycles were entirely absent in mice fed such a diet. On the other hand, the data recorded by other workers on the effect of a low lysine diet composed of 18 percent gliadin on estrus in rats indicate that occasional cycles are not unusual in some animals. In strain C3H virgin female mice fed the low lysine diet, both the appearance of mammary tissue in gross mounts and the observation of a few corpora lutea in the ovaries of some of the mice indicated that some estrous cycles occurred in some of the mice. This was in harmony with the finding of tumors in 25 percent of the mice. The reduction in tumor incidences from 97.4 to 25 percent and the lengthening of the average tumor age from 10.4 to 12.9 months were also in line with what might be expected. Indeed, it seems probable that if a change in the estrous rhythm is brought about by any means, an alteration in mammary-gland-tumor incidence in inbred highly susceptible mice, such as this subline of strain C3H, would occur.

SUMMARY

When fed a diet low in lysine from the time of weaning, 25 percent of virgin female strain C3H mice developed mammary-gland tumors at an average age of 12.9 months. Estrus in these mice was not completely absent.

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EFFECT OF CALORIC RESTRICTION ON MAMMARY-TUMOR FORMATION IN STRAIN C3H MICE AND ON THE RESPONSE OF STRAIN DBA TO PAINTING WITH METHYLCHOLANTHRENE

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In recent years evidence has been presented to indicate that there is a relationship between certain types of tumor growth and food intake. Rous (*1*) studied the effect of restriction of food intake on spontaneous mammary tumors in mice. He observed that after partial extirpation of these tumors recurrences resulted in 83 percent of the animals fed a normal diet ad libitum, while 41 percent were observed in those whose food intake was restricted. Furthermore, autotransplants of the excised tumors grew in 68 percent of the well-fed and in only 41 percent of the underfed mice. However, cures were not effected, since if those mice of the underfed group without recurrences or growing grafts were fed ad libitum, either growth of the graft, or recurrence of the partially extirpated tumor, or both, occurred soon after the host regained its normal weight. Bischoff, Long, and Maxwell (*2*) studied the effect of reduction in food intake upon the growth rate of inoculated sarcoma 180 in mice fed a commercial calf meal. A 50-percent food restriction resulted in considerable inhibition in growth of the transplants. Later Bischoff and Long (*3*) fed a constant but calorically insufficient amount of calf meal to all mice and supplemented the higher calorie groups with either starch or Crisco. This restriction in calories without a deficiency of essential nutrients also resulted in retardation of growth of mouse sarcoma 180.

Tannenbaum (*4*) used a diet composed of weighed amounts of dog chow meal and skim milk for both the calorie-restricted and full-fed mice. Enough cornstarch was added to the diet of the mice which ate ad libitum to make their caloric intake about double that of the restricted group. The mice were not kept in individual cages, but twice a week the mice fed a restricted diet in each group were sorted and caged according to body weight, so that the lighter mice did not have to compete with heavier ones for food. The number of both spontaneous mammary tumors and benzpyrene-induced skin tumors and sarcomas was lowered in the calorie-restricted mice. Visscher, Ball, Barnes, and Sivertsen (*5*) reduced dietary carbohydrate and fat to accomplish a one-third restriction in calories while the amount of protein, vitamins, and salts consumed in both restricted- calorie and ad libitum diets was the same. Virgin strain C3H female mice fed these diets showed a reduction in the incidence of spontaneous mammary tumors from 67 percent in the controls to zero in the calorie-restricted mice.

McCay (*6*), in an extensive study of the effects of nutrition on aging and longevity in rats, observed that restriction in caloric intake resulted in longer life span, due perhaps to reduction in most of the common rat diseases such as pneumonia, chronic nephritis, and various types of tumors, principally pulmonary lymphosarcomas.

Insufficient food consumption was, in the past, associated with suppression of estrus. Loeb (*7*), in work with guinea pigs, found that when the usual diet was fed in sufficiently restricted amounts to cause weight loss, the follicles of the ovary did not mature and frequently regressed. Evans and Bishop (*8*) found that when the food intake of rats was only slightly restricted, so that body weight did not exceed 200 gm., only 7 estrous cycles occurred in a year as compared with 55 in normally fed rats. If the diet was

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so restricted that body weight was held at 60–85 gm., no cycles were observed during a year. A similar study by Asdell and Crowell (*9*) confirmed these findings but added the further fact that estrous cycles were resumed when the severely restricted rats (body weight maintained at 40 gm.) were allowed enough food to gain 10 gm. in weight.

Work in this laboratory (*10*, *11*, *12*) indicated that diets which altered the occurrence of estrus lowered the incidence of spontaneous mammary tumors in a susceptible strain of mice. By feeding a diet so low in cystine that weanling mice were able to maintain their weight but could not grow, the incidence of spontaneous mammary tumors in strain C3H virgin female mice was reduced from 97 percent to zero (*10*). That this reduction was brought about by the suppression of estrus, at least in part, was shown by implanting pellets of diethylstilbestrol subcutaneously in female mice fed the low cystine diet (*11*). This resulted in mammary tumors in 45 percent of the animals. When a diet low in lysine, which did not suppress growth or estrus as completely as did the low cystine diet, was fed to strain C3H virgin female mice, the tumor incidence was 25 percent (*12*).

Young strain dba mice painted with an ether solution of methylcholanthrene developed leukemia in 90 percent of the cases when fed a normal diet. However, if they ingested the diet limited in cystine, the incidence of leukemia was reduced to 17 percent (*13*). That this reduction in the occurrence of methylcholanthrene-induced leukemia appeared to be related to the sulfur-containing amino acid content of the diet was shown by the fact that the incidence of leukemia was 72 and 78 percent in mice fed the low lysine and the lysine-supplemented diet, respectively (*14*).

This paper presents a study of the effect of a diet restricted in calories only on the incidence of the two types of tumors previously studied under other dietary regimens, namely, methylcholanthrene-induced leukemia in strain dba mice and spontaneous mammary tumors in strain C3H female mice.

EXPERIMENTAL PROCEDURE AND RESULTS

To study the effect of caloric restriction on mammary-tumor formation and development, strain C3H female mice, supplied by Dr. H. B. Andervont, of this Institute, were used. The virgin females, at weaning, were sorted into pairs of litter mates. One member of the pair was fed the complete diet ad libitum, while the other member received the restricted diet, the amount of which was governed by the food intake of the ad-libitum-fed animal on the preceding day. For every gram of food eaten by a mouse of the ad libitum group, 0.54 gm. of restricted diet was fed the following day to the paired litter mate in the calorie-restricted group. This amount of restricted diet contained the same amounts of casein, salts, yeast, and cod-liver oil as 1 gm. of the normal diet, but sufficiently less cornstarch and Crisco to reduce the calories 50 percent.

The diets used in this study are given in table 1.

TABLE 1.—*Diets fed mice to show the effect of caloric restriction*

Components	Normal diet [1]		Calorie-restricted diet	
	Grams	*Calories*	*Grams*	*Calories*
Casein [2]	18	81	18	81
Salts [3]	4		4	
Cod-liver oil	3	27	3	27
Yeast	10	35	10	35
Cornstarch	50	200	15	60
Crisco	15	135	4	36
Total	100	478	54	239

[1] Mice allowed to eat this diet ad libitum.
[2] Commercial grade from Casein Co. of America.
[3] Hubbell, Mendel, and Wakeman.

The breeding females, with the exceptions mentioned subsequently, were put on the experiment at 4 months of age after having borne and nursed one litter. These mice were sorted into littermate pairs and fed in the same way as the virgin females. Two pairs, or four

animals, were 6 months of age and had had two litters before the start of the experiment.

The mice were kept in individual cages. Food intake in the ad libitum group and food allotments for the restricted group were determined daily. Body weight was recorded semiweekly, at which time inspection was made for tumors. When a tumor was observed, a note of it was made in the record, and the mouse allowed to live about a week longer. All animals were necropsied. Sections of mammary-gland tumors were prepared, and the diagnosis was made by Dr. A. B. Eschenbrenner, of this Institute.

When the ad libitum member of a pair developed a tumor and was killed, an average of the last week or 10 days of normal food consumption was made; the restricted member of the pair was fed on the basis of this figure for the remainder of its life, unless the amount allowed was so low that a continual decline was evident. In this case an increase of 0.1 gm. of food per day was made to keep the animal from dying of starvation.

The effective number of mice is the number alive at the time of appearance of the first tumor. Where the number of mice in the restricted and ad-libitum-fed groups are different, some members of the restricted group had been unable to make the adjustment to a reduction of 50 percent in calories and had died. This was particularly true among the breeding females.

The average weight of the C3H virgin females at weaning was 14.9 gm. At 7 months of age the mice on the restricted diet weighed 13.7 gm., had an average length (nose to anus) of 8.9 cm., and were eating 1.54 gm. of restricted diet per day. The survivors of the ad-libitum-fed group at 7 months of age averaged 30.7 gm. in weight and 11.0 cm. in length. The breeding females of the restricted group at the start of the experiment weighed an average of 27.3 gm. After 3 months their weight dropped to 15.3 gm. The larger part of this loss took place during the first 2 weeks, and was nearly complete at the end of the first month. The ad libitum group of breeding mice weighed 25.9 gm. at the start, and the survivors 3 months later weighed 34.5 gm.

The results are summarized in table 2. The tumor incidence in both virgin and breeding ad-libitum-fed C3H females was 100 percent at an average age of 8.1 months. Among the calorie-restricted virgin females only 1 tumor arose at 13 months, giving an incidence of 12.5 percent. Two tumors, an incidence of 18.2 percent, developed among 12 calorie-restricted breeding females at 14 and 15 months. The 2 mice developing tumors were those which were 6 months of age and had borne 2 litters before being started on the experiment.

To study the effect of caloric restriction on leukemia induced by methylcholanthrene painting, strain dba mice were used. At weaning the mice were sorted into litter-mate pairs of the same sex. Twenty pairs, ten of males and ten of females, were used. The mice were individually caged and fed as described for strain C3H females in the mammary-tumor study. The mice were painted 25 times with a 0.2-percent methylcholanthrene solution in diethyl ether in a manner previously described (*13*) and were observed until death. All animals were necropsied. Sections were prepared, and diagnosis of leukemia was made as described by Mider and Morton (*15*). The average weight of ad-libitum-fed mice was 12.3 gm. at weaning and 23.7 gm. 60 days later. The calorie-restricted group weighed 13.6 gm. at weaning and 12.7 gm. 60 days later when they were consuming 1.37 gm. of restricted diet per day.

The results are summarized in table 3. In an average of 73 days from the time the methylcholanthrene painting was started, 96.2 percent of the ad-libitum-fed group had developed leukemia. In the calorie-restricted group, 35 percent developed leukemia in from 160 to 351 days, or in an average time of 237 days. The one case of arteriosclerosis occurred in a mouse without leukemia. Other neoplasms observed in the calorie-re-

TABLE 2.—*Effect of caloric restriction on mammary-tumor formation in virgin and breeding strain C3H female mice*

[Numerator = number of mice developing tumors; Denominator = number of mice dying free of tumor]

Group of mice	Diet	Effective number of mice	Mice developing tumors or dying free of tumors according to age (in months)														Total	Tumor incidence	Average tumor age
			6	7	8	9	10	11	12	13	14	15	16	17	18	22			
																		Pct.	*Months*
Virgin females	Ad libitum	11	2/0	4/0	1/0	1/0	2/0	0/0	1/0								11/0	100.0	8.1
Do	Restricted	10	0/0	0/0	0/0	0/0	0/1	0/0	0/0	1/0	0/0	0/1	0/3	0/0	0/1	[1] 0/1	1/7	12.5	13.0
Breeding females [2]	Ad libitum	21	2/0	4/0	8/0	3/0	4/0										21/0	100.0	8.1
Do [2]	Restricted	12	0/1	0/0	0/3	0/1	0/0	0/0	0/0	0/0	1/1	1/1	0/0	0/1	0/1	[3] 0/0	2/9	18.2	14.5

[1] 2 alive and tumor-free at end of 22 months.
[2] Had had 1 litter before being put on experiment. Two pairs, including the 2 in the restricted group which developed tumors, had had 2 litters and were 6 months of age before being put on the experiment.
[3] 1 alive and tumor-free at end of 22 months.

stricted group were: epidermoid carcinoma of the skin in four mice, papilloma of the skin in two, and one each of subcutaneous fibrosarcoma, hemangioma (ovary), and granulosa-cell tumor of the ovary.

TABLE 3.—*Effect of caloric restriction on the response of strain dba mice to painting with methylcholanthrene*

Diet	Number of mice	Mice with leukemia [1]			Mice with other malignant neoplasms [2]		Total mice with malignant neoplasms	
		Number	Percent	Mean latent period	Number	Percent	Number	Percent
				Days				
Ad libitum	[3] 26	25	96.2	73			25	96.2
Restricted	20	7	35.0	237	5	25.0	12	60.0

[1] Pathologic diagnosis described by Mider and Morton (*15*).
[2] Epidermoid carcinoma in 4 mice, and subcutaneous fibrosarcoma in 1 mouse.
[3] 6 mice had no paired litter mate in the restricted group.

DISCUSSION

In this subline of strain C3H mice, which normally shows a 97.4-percent incidence (*16*) of mammary tumor in either virgin or breeding females, caloric restriction reduced the incidence to 12.5 percent. Vaginal smears were made daily for 2 months from the calorie-restricted group of virgin females when they were 7 and 8 months of age. There was no evidence of estrus in any of the mice. A gross mount (*17*) of mammary tissue from the one mouse in which a tumor occurred, revealed slight duct development, no budding along the ducts, and only occasional terminal buds, so that even in this mouse estrous cycles were very few.

The young mice of both strain C3H and dba which were fed the calorie-restricted diet from weaning did not grow (gain in body weight); neither did strain C3H mice grow when fed a low cystine diet, which was shown to prevent mammary tumors (*10*). The question might be raised whether this failure of the whole animal to grow was not a possible explanation of the suppression of mammary-tumor development. However, the fact that strain C3H mice, fed a low cystine diet from weaning but bearing a subcutaneously implanted pellet of diethylstilbestrol, did develop tumors in 45 percent of the cases, shows that lack of growth of the potential host in itself was not the complete explanation of failure of tumor development.

Furthermore, methylcholanthrene-painted strain dba mice fed either a low cystine or a low lysine diet did not grow (*13*, *14*). Nevertheless, while the incidence of leukemia was reduced from 90 percent in mice fed a cystine-supplemented diet to 10 percent in mice fed the low cystine diet, the incidence

of leukemia in mice fed the high lysine diet was 78 percent, and in those that ingested the low lysine diet, 72 percent. Thus again it was found possible for tumors to form and grow to the point where they were easily detected in hosts which, because of dietary restrictions in single essential amino acids, were unable to increase their own body weight.

The mice which ingested diets restricted in single essential amino acids ate at least as much food per gram of body weight as those fed diets supplemented with the lacking amino acid. In view of this fact, it might be that the changes in tumor incidence brought about by amino-acid deprivation and those reported in this paper, in which the effect of caloric restriction was studied, may have been the result of fundamentally different alterations in physiologic processes.

While the most probable mechanism of cancer suppression by caloric restriction in the case of mammary-gland tumors was through the reduction or abolishment of estrous activity, this could not have been the primary mode of action in methylcholanthrene-induced leukemia. Of the seven calorie-restricted strain dba mice developing leukemia following methylcholanthrene painting, three were females, and four males.

It seems probable that all of the mice 50 percent restricted in calories must have adjusted their metabolic needs to a lower level in order to survive. This adjustment probably was mediated through the thyroid gland. In completely thyroidectomized animals, the basal metabolic rate falls about 40 percent. The restriction in this experiment was 50 percent, so that the ability of the mice to lower their caloric needs must have been pressed to the utmost. Such an adjustment would suppress sexual activity in the female but would alter many other processes, among which are those concerned in the development of methylcholanthrene-induced leukemia.

SUMMARY

A 50-percent reduction in calories, without alteration in the amount of dietary essentials, reduced the incidence of mammary-gland tumors from 100 to 12.5 and 18.2 percent in strain C3H virgin and breeding females, respectively.

In methylcholanthrene-painted strain dba mice, 50-percent caloric restriction caused a reduction in the development of leukemia from 96.2 to 35 percent, and an extension in mean latent period of from 73 to 237 days.

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EFFECT OF DIET UPON THE DEVELOPMENT OF MAMMARY GLANDS IN STRAIN C3H MICE

By FLORENCE R. WHITE, *assistant biochemist, National Cancer Institute, National Institute of Health, United States Public Health Service*

The full growth of the mammary gland has been shown to be under the control of several hormones (*1*, *2*). However, for full virginal development, the estrogenic hormone is of primary importance. Since partial or complete suppression of estrus results in little elaboration of estrogenic hormone by the ovary, the effects of such suppression should be evident in the appearance of the mammary glands.

Strain C3H virgin female mice became anestrus when fed a low cystine diet. Mice so fed did not develop any mammary tumors, although normally fed mice of this strain showed a 97.6-percent tumor incidence (*3*). A subcutaneously implanted pellet of diethylstilbestrol raised the tumor incidence of mice ingesting the low cystine diet to 44.7 percent (*4*). An alteration of tumor incidence and of estrus in strain C3H female mice was also brought about by a diet low in lysine (*5*) and by caloric restriction (*6*). This paper shows how the development of the mammary glands was affected by these dietary regimens.

EXPERIMENTAL PROCEDURE

Gross mounts of mammary tissue from over 200 strain C3H female mice of the Andervont subline were prepared. Although the mice were part of experiments set up for other purposes, in all cases they were killed when a mammary tumor was detected, or when death seemed imminent from some cause apart from a mammary tumor. The second or third thoracic mammary gland from either the right or the left side, whichever was without a tumor, was fixed, dissected out in its facial layer, stained with hematoxylin, and mounted in balsam. Preparations from some of the mice on each diet were photographed, and representative ones selected for the figures in this paper.

The mice, with the exceptions noted subsequently, were virgin females which were fed the diet specified from weaning until death. One group of breeding females was maintained on Purina dog chow until they were 4 and 6 months old and had borne and nursed one and two litters, respectively, before the experiment was begun.

Diets fed the mice were low cystine (*3*), cystine supplemented (*3*), dog chow, low lysine (*5*), a normal diet eaten ad libitum by the mice (*6*), and a comparable diet fed so that the caloric intake was restricted (*6*).

RESULTS

The mammary glands of virgin female mice fed a low cystine diet from weaning were rudimentary. Figure 1, *A*, shows a typical preparation. The gland remained small (this illustration shows very nearly the whole of the third mammary gland), and there was little branching of the ducts, and no acini development. No tumors were found among such animals (*3*). However, mice fed the low cystine diet, but with a subcutaneously implanted pellet of diethylstilbestrol, had mammary glands (fig. 1, *B*) in which the duct system had become complex through much branching, and acini had developed along the sides of the ducts and in clusters at the duct ends. The glands were similar to those of virgin females fed a cystine-supplemented diet, chow, or a complete diet ad libitum in which full development of the extent and complexity of the duct system had taken place. The tumor incidence in mice bearing diethylstilbestrol pellets and fed a low cystine diet was 45 percent (*4*), and that in mice fed chow or other complete diets was from 97 to 100 percent (*3*, *6*).

Mammary tumors developed in 25 percent of the virgin female mice fed a low lysine diet (*5*). Estrus was thought to occur occasionally among at least some of these mice, because a few

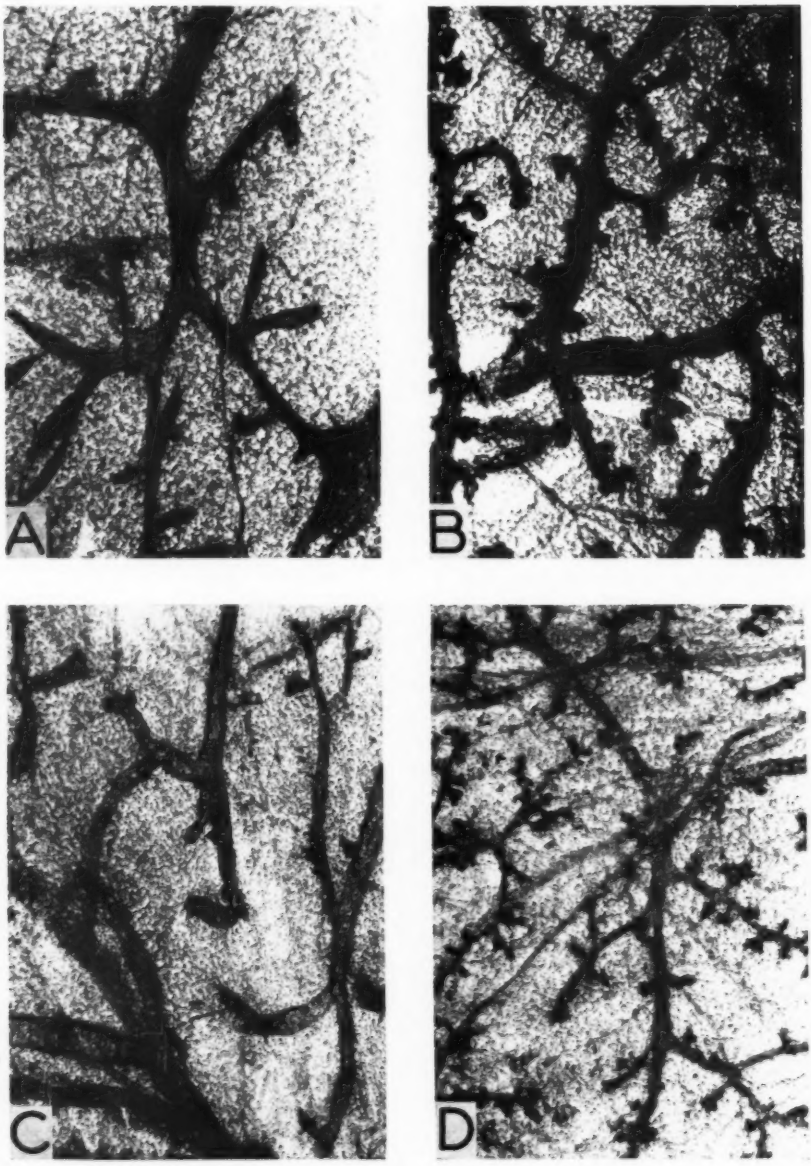

FIGURE 1.—Gross preparations of mammary glands, all from virgin female mice. *A*, From mouse 11 months old fed a low cystine diet; *B*, From a mouse 8 months old fed a low cystine diet, with subcutaneous stilbestrol pellet; *C*, From a mouse 18 months old fed a low lysine diet, no tumor development; *D*, From a mouse 14 months old fed a low lysine diet, with tumor development. See text. × 20.

corpora lutea were often present in the ovaries at necropsy. Figure 1, *C*, shows the mammary gland from a mouse which was fed a low lysine diet and which did not develop a tumor. There was a little growth of the duct system, but no development of acini. Figure 1, *D*, mammary tissue from a mouse, which was fed the same diet and which did develop a mammary tumor, shows rather extensive development of the duct system and acini. The mammary tissue in the preparation had probably undergone partial atrophy. This might have been due to a prolonged period of anestrus immediately preceding the death of the animal. In this case, the estrus which had evoked the growth shown by this mammary preparation took place sometime earlier.

Figure 2, *A*, shows the mammary-gland preparation of a virgin female and figure 2, *B*, of a breeding female which ingested a complete diet ad libitum. Tumor incidence was 100 percent in both groups of mice (*6*). While there was variation from one animal to another, these two illustrations were representative of full virginal and of full nonsecreting parous development. The extent and complexity of the duct system was not greatly different in the two, but there was usually greater acini development in the glands from the breeding females.

When a diet complete in all the dietary essentials but 50 percent restricted in calories was fed to virgin C3H female mice from weaning, the mammary-tumor incidence was reduced from 100 to 12.5 percent (*6*). Figure 3, *A*, shows the mammary gland of a calorie-restricted mouse which did not develop a tumor. The duct system remained rudimentary, showing little if any growth or branching. There was no development of acini. Figure 3, *B*, is also from a calorie-restricted animal, but this mouse did develop a mammary tumor. There is evidence of only a slight amount of ductal development. Figure 3, *C* and *D*, are of the mammary glands of breeding C3H female mice.

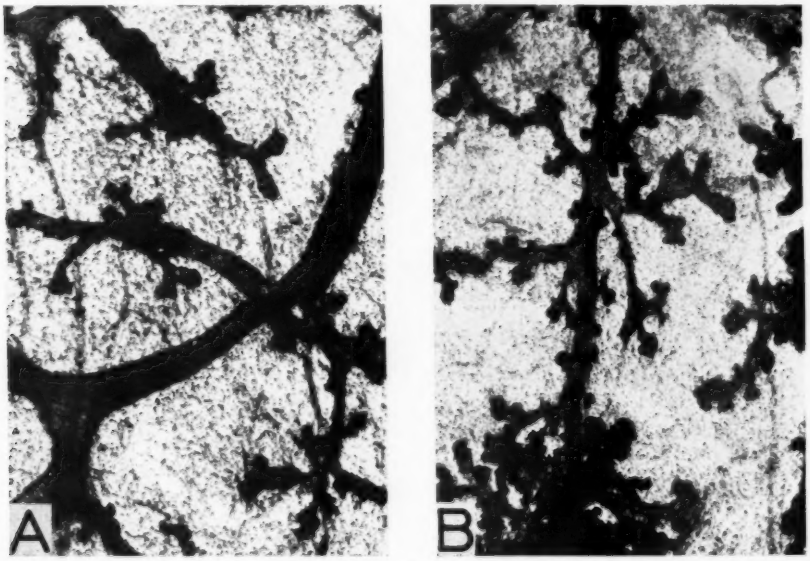

FIGURE 2.—Gross preparations of mammary glands. *A*, From virgin female 12 months old which ingested a complete diet ad libitum; *B*, From breeding female 10 months old, which ingested a complete diet. × 29.

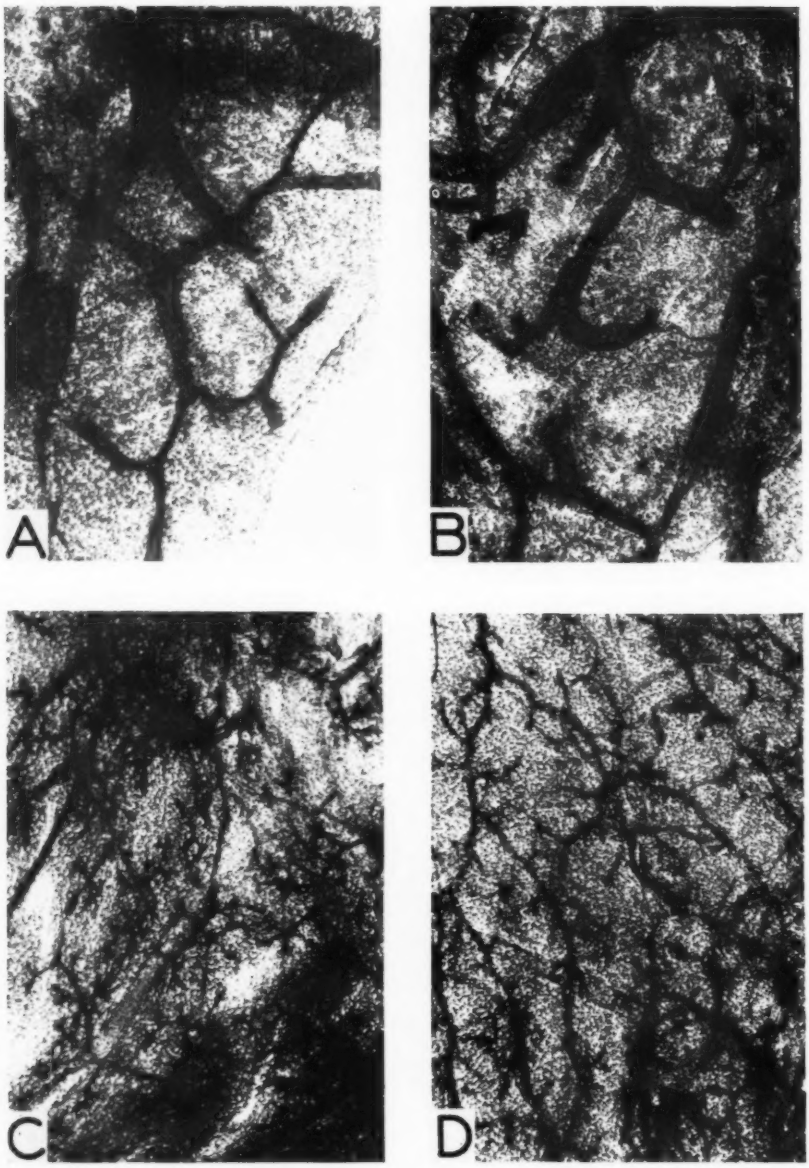

FIGURE 3.—Gross preparations of mammary tissue. *A*, From a virgin female 8 months old, caloric restriction, no tumor development; *B*, From a virgin female 13 months old, caloric restriction, with tumor development; *C*, From a breeding female 8 months old, caloric restriction, no tumor development; *D*, From a breeding female 14 months old, caloric restriction, with tumor development. × 29.

Caloric restriction was started when they were mature and had borne and nursed one or two litters. The mammary tissue in figure 3, *C*, was from a mouse 4 months old which had borne one litter, and that in figure 3, *D*, was from a mouse 6 months old which had had two litters, when the experiment started. A tumor developed in the latter mouse but none in the former. The glands of both showed atrophy of a fully developed mammary gland, similar to what would be expected if the ovaries had been removed after adult development had taken place. It seemed possible that the transformation to the malignant state may have occured in one (fig. 3, *D*) before the caloric restriction was begun.

SUMMARY

Mammary tissue of strain C3H virgin female mice fed a low cystine diet showed an absence of growth which indicated that estrus had been suppressed. Mice fed the same diet but kept in continuous estrus by a subcutaneously implanted pellet of diethylstilbestrol had mammary tissue in which considerable growth had taken place.

Mammary tissue of virgin female mice fed a diet low in lysine showed variations from very little to considerable growth, which indicated that in some of the mice estrus had occurred, while in others it had been absent.

Among the virgin female mice restricted in calories, mammary tissue showed little evidence of growth. Mammary tissue from breeding females fed the same diet was atrophied, which indicated that sexual activity had been stopped by the caloric restriction.

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CHEMICAL STUDIES ON HUMAN CANCER. I. CYTOCHROME OXIDASE, CYTOCHROME *C*, AND COPPER IN NORMAL AND NEOPLASTIC TISSUES[1]

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INTRODUCTION

Up to the present time, the only method of characterizing neoplastic tissues and of distinguishing them from normal tissues has been by microscopic examination, i. e., by morphologic criteria. This method is still the most unequivocal in the recognition of neoplasia and indeed in the recognition of degrees of neoplasia. In the past few years, attempts have been made to supplement the morphologic criteria of altered tissues with characterizations based upon chemical reactions in vitro, chiefly enzymatic, which such tissues reveal in the presence of known and added substrates (*1–16*). Since the chemical studies possess a number of variations in the approach to this problem of characterization, they not only have a descriptive value but also contain the possibility of discovering at what point or points in the numerous chemical processes of a given tissue the deviation toward the neoplastic state occurs. While the chemical approach to the study of neoplastic tissues is potentially of great power, it can never be separated from a consideration of the gross pathology of the tissues and of their microscopic morphology, for these last-mentioned criteria serve as the foundation upon which the chemical studies are based, and by which they are largely interpreted.

Chemical studies on neoplastic tissues have so far been performed almost entirely with laboratory animals. These studies possess an unquestioned value and many advantages, but their limitations are obvious. The essential core of experimental cancer investigation is a better understanding of the nature of malignancy in man. Work on experimental animals, limited to the number of tumors available, may point the way to this understanding, but there is no assurance that the findings may be applicable to human beings. Studies on the chemical characterization of normal human tissues are rare, on human tumors they are extremely few, fragmentary, and largely uncoordinated because of the lack of comparable studies on normal human tissues. The only clear-cut study of human tissue so far (*10*) has involved the investigation of a single chemical component in the normal kidney cortex, colonic mucosa, and lung, and in six adenocarcinomas of the gastrointestinal tract.[2] Neoplastic growths in man possess a wide range of morphologic and pathologic characteristics, even in the same type of growth; not only are they highly variable, but they may be observed to arise in forms so far not observed in experimental animals. Although certain kinds of benign tumors are known to occur in laboratory animals, the range between entirely benign and highly malignant growths in man has gradations and greater diversity and complexity than that so far observed in the lower animals. Studies on human neoplastic growths, therefore, require a

[1] This paper is a joint contribution from the National Cancer Institute and the Memorial Hospital for the Treatment of Cancer and Allied Diseases (New York City). The cooperative study was made possible originally by arrangements made by Dr. R. R. Spencer, of the National Cancer Institute, and Dr. C. P. Rhoads, of the Memorial Hospital. Subsequent arrangements to obtain other pathologic material from the New York Hospital were made through the kindness of Dr. G. B. Mider, of the Cornell University Medical College. Tumor material was supplied through the kindness and cooperation of Dr. Fred W. Stewart, of Memorial Hospital, and Dr. N. C. Foot, of New York Hospital.

[2] Consideration is omitted here of the vast amount of work on human blood and serum in the course of a wide variety of pathologic states, including cancer, of the host. Studies of systemic effects evoked in human tissues in sites removed from that of the cancer, such as the recent investigations of the liver functions by Rhoads, Pack, Abels, and their associates at the Memorial Hospital (*17*) are likewise not considered here.

large variety of such tissues, together with comparable studies on the widest possible selection of normal human tissues.

Of the various chemical approaches to the study of malignancy in tissues, the solution of the problems concerned with metabolism and respiration ranks among the most important. The complex and interrelated functions by which living tissues are maintained require the ready availability of sources of energy. To some extent, these sources of energy are supplied by the oxidation of substances of relatively low molecular weight, amino acids, sugars, and fatty acids. In primitive forms of life, the energy derived from the oxidation of these metabolites may be obtained entirely under anaerobic conditions (fermentation). In the higher animals there are present, in addition to anaerobic systems, oxidation mechanisms whereby molecular oxygen is utilized (respiration) (*18*). Most of the tumors which have been studied appear to obtain a considerable part of their energy from fermentative processes (*1*, *6*), although they exhibit considerable respiration. The study of this anomalous phenomenon requires a knowledge of the individual tissue systems concerned with both anaerobic and aerobic types of metabolism. In this paper, attention is limited to certain factors related to the utilization of oxygen by the various tissues studied.

The activation of molecular oxygen for the purposes of intracellular oxidation is performed in part by iron-containing, cyanide-sensitive, catalytic systems which are capable of existing reversibly in reduced (ferrous) and in oxidized (ferric) states. Knowledge of these systems is by no means complete; but the best available data indicate that they are composed of several components each of which is a conjugated protein containing a hemin nucleus as the prosthetic group. Each of the components, when in the reduced condition, gives sharply defined absorption bands in the visible region of the spectrum, and by this means has been separately identified (*3*, *19*). The generic term for these components is "cytochrome," and the various components have been given the designations cytochrome oxidase, cytochrome *a*, cytochrome *b*, cytochrome *c*, etc. (*19*). Only two of these components have so far been isolated in relatively pure form, cytochrome oxidase (*2*, *20*, *21*) and cytochrome *c* (*22*), and the mode of action of only these substances of all the cytochromes is understood at least partially. The main cyclic pathway by which these components utilize molecular oxygen in the oxidation of a given metabolite is as follows:

$$\text{Molecular } O_2 + \text{Reduced cytochrome } c \xrightarrow{\text{Cytochrome oxidase}} \text{Oxidized cytochrome } c \qquad (1)$$

$$\text{Oxidized cytochrome } c + \text{Metabolite} \longrightarrow \text{Oxidized metabolite} + \text{Reduced cytochrome } c \qquad (2)$$

Molecular oxygen thus does not act directly upon the metabolite but only indirectly through the mediation of the cytochrome oxidase-cytochrome *c* system. Neither cytochrome *c* nor cytochrome oxidase is autoxidizable; taken together, the two components will cause an uptake of oxygen if cytochrome *c* is in the reduced state.

The normal tissues of animals contain the cytochrome oxidase-cytochrome *c* system to greater or less degree, heart muscle being by far the most active, liver and kidney intermediate, and spleen and lung among the least active (*2*, *5*). In all the normal tissues studied, the activity of cytochrome oxidase and the concentration of cytochrome *c* run roughly parallel (*2*, *5*). The study of this system in the neoplastic tissues of laboratory animals has been well-done but has been limited by and confined to the relatively few types of tumors available in rats and in mice. The results obtained indicate that, in comparison with such highly active tissues as heart and kidney, the activity of the tumors is low; on the other hand, the activity of the tumors is of the same order of magnitude as that

of such tissues as spleen and lung. The activity of cytochrome oxidase has never been reported for human tissues whether normal or neoplastic. There is a single report in the literature on the cytochrome *c* concentration in three normal human tissues and in six neoplasms (*10*). It was considered desirable, therefore, to investigate these systems in the wide variety of neoplasms offered by the clinic, and to make comparable studies of normal tissues available through the medical examiner's office. It has been suggested (*19*, *23*) that the activity of cytochrome oxidase in tissues is connected with the presence of copper, and for this reason copper determinations were made on many of the tissues studied.

In no sense can the studies herein described be considered complete. The full characterization of any tissue in terms of its content of catalytic systems requires the amassing of complete data on a large number of representative systems (*13*). A tissue low in certain systems may be high in others (*16*). Only if the full "spectrum" of enzymatic activity of a given tissue is known can the tissue, in this sense, be said to be described. For these reasons alone, the data given in the present paper on the cytochrome oxidase-cytochrome *c* system in human tissues represent only a small part of the diverse and complicated pattern of enzymatic catalysis.

EXPERIMENTAL PROCEDURE

NEOPLASTIC HUMAN TISSUES

Nearly all the tumors studied were obtained after operation. Those which were not so obtained were received on necropsy, and in these cases metastases were primarily selected. Care was taken to avoid any contact of these tissues with the ever-present formaldehyde of the pathology laboratory. The time which elapsed between the excision of the tumors in the operating room and the receipt of these tissues in our hands varied from ½ hour to 2 hours. The necropsy material was obtained in 2 to 20 hours after death. All the surgical specimens came from the Memorial Hospital and the New York Hospital. The hospital numbers of the specimens with the prefix M designate the former hospital and those with the prefix C the latter. One necropsy specimen was obtained from the Morrisania Hospital through the kindness of Dr. Aronson. The specimen from this hospital is designated by the prefix Morr. —. The clinical and radiation records were examined of all the patients from whom tissues were obtained; these were recorded but not further discussed as they had no apparent bearing on the results obtained. Sections of all the tissues obtained were preserved in formalin.

NORMAL HUMAN TISSUES

Samples of various tissues were obtained directly at necropsy on persons dead of accident or by self-inflicted means. All materials were obtained from the medical examiner's office of Queens County. The various persons, the cause of death, and the time elapsed between death and necropsy (when the tissues were received) are as follows:

H. v. S.—Male, white, 57 years old, death due to coronary occlusion, necropsy performed 1 hour after death; hospital number A2645.

D. M.—Female, white, 58 years old, death due to rupture of sigmoid colon, necropsy performed 1 hour after death; hospital number A2647.

A. P.—Male, white, 41 years old, death due to skull fracture, necropsy performed 3 hours after death; hospital number 43RA22M.

T. K.—Male, white, 50 years old, death due to carbon monoxide inhalation, necropsy performed 4 hours after death; hospital number 44A35–M.

A. R.—Male, white, 61 years old, death due to cerebral hemorrhage, necropsy performed 3 hours after death; hospital number A2786–M.

IMMEDIATE TREATMENT OF THE TISSUE

On two separate occasions, two of the present authors (J. P. G. and F. M. L.) spent some time in the laboratories of the Memorial Hospital. In the course of their first stay in New York, all the tissues obtained, both normal and neoplastic, were placed immediately on receipt in covered individual glass containers and set in finely ground solid carbon dioxide (dry ice) which froze the tissues within 5 minutes. All tis-

sue specimens from Memorial Hospital, which are prefixed with the letter Q, were treated in this way, as were the New York Hospital specimens Nos. 73770, 370167, 365663, 370323, 369537, 336902, 327819, 370367, 76414, 371203, 369671, 370369, 370059, 319745, 371643 as well as the specimens from the Morrisania Hospital, and the normal tissues from H. V. S., D. M., and A. L. All these specimens were kept during the period of collection in an insulated wooden box containing finely chopped dry ice in a cold room. At the end of this period, the box still containing dry ice was rapidly transported by truck to Bethesda and set in a cold room in the National Cancer Institute. Studies on these frozen tissues were then performed at leisure at the Institute.[3] No tissue was ever allowed to thaw except immediately prior to its study.

In the course of the second stay of the authors (J. P. G. and F. M. L.) at the Memorial Hospital, the tissues were studied at the hospital immediately on receipt. Tissues thus studied in the fresh state included surgical specimens from the Memorial Hospital the numbers of which are prefixed with the letter R, surgical specimens from the New York Hospital Nos. 374195, 379946, 350370, and normal tissues from T. K. and A. R.

The pathologic diagnoses were taken from the records of the hospitals. In the case of certain of the tumors, the percent of epithelial and of pigmented areas was determined by the method of Chalkley (*24*).

METHODS OF INVESTIGATION

CYTOCHROME OXIDASE

The tissues were carefully trimmed of extraneous matter, care being exerted to remove any necrotic area from the tumors. For the determinations of cytochrome oxidase, 1 gm. of each tissue was transferred to a glass mortar and thoroughly ground with sand and 10 cc. of 0.2 M phosphate buffer at pH 7.2. After the sand was allowed to settle, the suspension served as the source of the oxidase.

The activity of cytochrome oxidase was determined by measurement of the rate of oxygen consumption of the suspensions in the presence of an excess of cytochrome *c* and of a substrate which reacts directly with cytochrome *c*, in this case *p*-phenylenediamine. Under the conditions chosen, the oxidase was the limiting factor (equations 1 and 2). The oxygen consumption was measured with single Warburg manometers. The flasks contained 1 cc. of the tissue suspension (equivalent to 100 mg. wet weight of tissue), 1 cc. of phosphate buffer at pH 7.2, and 1 cc. of cytochrome *c* solution. The cytochrome *c*, which was prepared from veal hearts by the method of Keilin and Hartree (*22*), had a concentration of 1×10^{-4} M. The side arm of the flask contained 0.3 cc. of freshly prepared 2 percent *p*-phenylenediamine. After equilibration at 38° C., the contents of the side arm were mixed with the cell contents; and the manometers were read at 5-minute intervals for 20 to 30 minutes, during which period the oxygen consumption was linear with time. The hourly oxygen consumption per milligram of wet weight of tissue was calculated from this period.

Inasmuch as *p*-phenylenediamine reacts with other components of the cytochrome group, namely, the autoxidizable cytochrome *b* (*25*), corrections were made by measuring the uptake of oxygen by a mixture wherein 1 cc. of 0.01 M potassium cyanide (KCN) was substituted for the 1 cc. of cytochrome *c*. This blank therefore represents the oxidation of *p*-phenylenediamine and of any other substrates in the tissue through autoxidizable, cyanide-insensitive systems. The activity of cytochrome oxidase is thus given as:

$$Qc = \frac{\text{Cubic millimeters of } O_2 \text{ (in the presence of cytochrome } c\text{)} - \text{Cubic millimeters of } O_2 \text{ (in the presence of KCN)}}{\text{Milligrams wet weight of tissue} \times \text{hours}} \quad (3)$$

[3] The period of collecting the frozen tissues in New York was 3 months. The subsequent study of these tissues in Bethesda, which immediately followed this period, lasted another 3 months. The average length of time that any one tissue was kept in the frozen state before being studied was 4 months.

The correction factor obtained in the presence of cyanide was negligible in all cases except those involving the melanotic melanomas. Here the consumption in the presence of cyanide was appreciable. This point is discussed later.

A number of the tissue suspensions, both normal and neoplastic, were studied in the absence of added cytochrome *c*. In these cases, the rate of oxygen consumption was measured of mixtures of 1 cc. of the suspension plus 2 cc. of buffer at pH 7.2 with subsequent addition of the above-mentioned amount of *p*-phenylenediamine from the side arm of the flask. This rate was invariably lower than that observed in the presence of an excess of added cytochrome *c* and afforded a measure of the over-all cytochrome oxidase-cytochrome *c* activity in the tissue suspension. As was to be expected, when 1 cc. of 0.01 M potassium cyanide solution was substituted for 1 cc. of buffer in these mixtures, the rate of oxygen uptake was too low to measure, with one notable exception, the melanotic melanomas.

All determinations were run in duplicate. The duplicates agreed within values of 5 percent. Different samples of the same tissue agreed with each other in the range of ±10 percent. In view of the fact that the tissues were employed in heterogeneous suspension, not in homogeneous solution, the agreement found between replicate determinations is satisfactory.

CYTOCHROME *C*

For the determination of cytochrome *c* in the normal and neoplastic tissues, 5 to 10 gm. of tissue was carefully weighed, transferred to a glass mortar, and thoroughly ground with sand and water. The cytochrome *c* was isolated from the aqueous extracts and measured by a standardized veal heart-cytochrome oxidase preparation according to the method of Stotz (*2*).[4] The oxidase was standardized every day against various known concentrations of cytochrome *c*. The autolytic procedures for the standard oxidase preparation suggested by Haas (*21*) were employed. The preparation under these conditions maintains a nearly constant activity over several weeks' standing in the ice box. The sensitivity of the method was frequently checked by addition of cytochrome *c* to the mixtures; the recovery was invariably 80–90 percent. Because of the limited amount of each neoplastic tissue, few duplicate determinations per sample could be made; but where these determinations were made, they usually agreed to within 25 percent.

In the method of determining cytochrome *c*, this substance is the limiting factor, for under the conditions employed cytochrome oxidase is present in excess.[5] The concentration of cytochrome *c* in the various tissues is given in terms of milligrams per 100 gm. of wet tissue.

COPPER

The determination of copper in tissues has been described (*26*). The method is colorimetric and depends upon the use of diethyldithiocarbamate. Recovery tests on added copper sulfate to several tissue digests revealed that 90–100 percent of the copper was recoverable by the methods used.

CONTROL STUDIES ON RAT TISSUES

It is possible to study animal tissues immediately after removal from the body of the animal. For many obvious reasons, it is difficult to apply this ideal condition to the study of human tissues, particularly those obtained from normal subjects. Effort was made in the present studies to collect both normal and neoplastic tissues in as fresh a condition as possible, but an appreciable interval of time, ranging from half an hour to 2 hours after operation and from 1 hour to 4 hours after death, elapsed before the neoplastic and the normal tissues, respectively, were obtained. Furthermore, the greater num-

[4] *p*-Phenylenediamine was used here instead of hydroquinone. The high rate of autoxidation of the latter is a serious handicap in its use.

[5] Methods involving spectroscopic determinations of cytochrome *c* have been reported by several investigators (*4, 10*).

ber of these tissues were kept in a frozen state in solid carbon dioxide for several months before being studied. It is believed that freezing under these conditions has no effect on the catalytic systems studied because (1) it has been shown that the activity of cytochrome oxidase is unimpaired after repeated freezings and thawings (*20*), (2) crystalline proteins with well-defined catalytic properties have been isolated from tumor tissue (Jensen rat sarcoma) after the tissues had stood in the frozen state for several months (*27*), and (3) the activity of cytochrome oxidase and the concentration of cytochrome *c* is of the same order of magnitude in the same human tissues studied either in the fresh or the frozen state. (See tables.) One of the normal human subjects died of carbon monoxide inhalation, but his tissues yielded catalytic activities indistinguishable for the most part from those obtained on the tissues of normal subjects dead of accidental causes. Although it might appear that the freezing of the tissues, and the inhalation of carbon monoxide may be ruled out as factors affecting the particular catalytic properties of the tissues studied, the extent of the factors involved in the interval of time during which the tissues lie either on the pathologist's table or within the cadaver is still unknown. For this reason, it was considered desirable to investigate the catalytic activities of the tissues of an experimental animal, the rat, in the fresh condition and under conditions approximating those observed when working with human tissues. Some animals were killed by decapitation, others by carbon monoxide inhalation. The tissues of some animals were removed and studied immediately; the tissues of others were removed, allowed to stand for about an hour at room temperature, frozen in dry ice for 2 months, and then studied; whereas the carcasses of still others were allowed to stand at room temperature for 2–4 hours and the tissues then removed and studied. The room temperature varied from 20° to 23° C. The animals dead of carbon monoxide asphyxiation were among those whose carcasses were allowed to stand for the 4-hour period before the tissues were removed for study. The data are given in table 1.

TABLE 1.—*Cytochrome oxidase activity and cytochrome c concentration in fresh, standing, and frozen rat tissues* [1]

Tissue	Condition of tissue					
	Fresh [2]		Standing 4 hours [3]		Frozen 2 months [4]	
	Cytochrome oxidase (Q_{O_2})	Cytochrome *c*	Cytochrome oxidase (Q_{O_2})	Cytochrome *c*	Cytochrome oxidase (Q_{O_2})	Cytochrome *c*
		Milligrams pct.		*Milligrams pct.*		*Milligrams pct.*
Liver	11.8 (6.3) [5]	8.6	12.0 (7.8) [5]	9.2	11.5	8.6
Spleen	4.3	.....	4.0	.....	4.4	.....
Kidney	14.2	.....	12.3	.....	15.4	.....
Heart	37.4	.....	40.2	.....	42.8	.....
Brain	12.5	.....	14.8	.....	.....	.....
Skeletal muscle	6.4	.....	6.2	.....	.....	.....
Jensen sarcoma	1.6 (.24) [5]	1.2	1.5 (.20) [5] [6]	1.1	1.7	.....

[1] All data given on basis of tissue wet weight; Buffalo rats used. Values are averages of several animals. Q_{O_2}=Cubic millimeters 0_2 per milligram per hour.

[2] Animals were decapitated and tissues immediately removed for study.

[3] Animals either decapitated or killed by carbon monoxide inhalation and carcasses allowed to stand 4 hours at 20°–23° C. before the tissues were removed.

[4] Animals decapitated; tissues were removed and stored immediately in dry ice, kept for 2 months, and then studied.

[5] Data in parentheses refer to experiments in which buffer was substituted for cytochrome *c* solution (*p*-phenylenediamine present).

[6] Animals decapitated; tumors removed immediately and allowed to stand 2 hours at room temperature before study.

The data in table 1 indicate that the periods of standing at room temperature or at dry-ice temperature have little observable effect upon the systems studied; the values for the tissues studied under these conditions are very nearly the same for the same tissues obtained immediately from the freshly killed animal. The inhalation of carbon monoxide also appears to have little if any effect upon the catalytic systems in these tissues. This finding is discussed more fully later. The fact that the freshly obtained tissues of rats gave values similar to those obtained on tissues which had been kept standing in different ways is no guarantee that hu-

man tissues would behave in the same manner, but at least it allows for the probability that they would and permits of a certain degree of confidence in the results with the latter tissues.[6]

The values in parentheses in table 1 represent the rate of oxygen consumption by certain of the tissue suspensions in buffer and in the presence of *p*-phenylenediamine. These values, at least for liver, are an appreciable proportion of the oxygen consumption by the suspension in the presence of excess cytochrome *c* (cytochrome oxidase activity).

The relative values of cytochrome oxidase for the rat tissues are similar to those observed by other investigators although the absolute values reported here are of a generally lower order of magnitude (*2*, *5*, *9*). The value for the concentration of cytochrome *c* in rat liver is in good agreement with that reported by Stotz (*2*) and by Shack (*9*); that for the same component in the Jensen rat sarcoma is similar to the value reported by Du Bois and Potter (*4*).

CYTOCHROME OXIDASE, CYTOCHROME *C*, AND COPPER IN NORMAL AND NEOPLASTIC HUMAN TISSUES

The data on cytochrome oxidase, cytochrome *c*, and copper in normal and neoplastic human tissues are given in table 2.

There is substantially good agreement among the values for each tissue of the five normal persons studied; and this agreement appears to hold, independently of the manner of death, or whether the tissues were studied immediately after necropsy or after a period of standing in the frozen state. For this reason, also to conserve space, only the average values with the variations for each tissue are presented. The normal values of cytochrome oxidase and of cytochrome *c* in the tissues of T. K., who died of carbon monoxide poisoning, are of considerable interest and have been confirmed by similar studies on rat tissues (table 1). The respiratory activity of tissues may be suppressed by saturation of the iron-containing systems with carbon monoxide (*28*, *p. 74*), but this inhibition takes place only in the dark and is reversible on illumination. Combination of carbon monoxide with hemoglobin, on the other hand, is of a firm and irreversible character. It seems probable that death from carbon monoxide inhalation occurs by displacement of oxygen from hemoglobin with subsequent asphyxia; the large amount of hemoglobin present in the circulating blood would presumably combine with a lethal amount of the gas before it could diffuse into the tissues. However, if some of the gas escaped into the tissues and there combined with the respiratory iron systems, the effect would not be observed because of the subsequent manipulations of the excised tissues in the presence of light.

One of the most striking observations drawn from the data on cytochrome oxidase and cytochrome *c* (table 2) is the relatively low value of all the tissues in comparison with the same tissues in the rat (table 1). The general order of activity of the tissues in man is more or less similar to that of the rat; e. g., heart muscle is by far the most active, liver, kidney and brain are intermediate, while spleen and lung are among the lowest. Among the muscles, the catalytic activity in descending order is as follows: heart, diaphragm, skeletal, and bladder. Smooth muscle in general, which would include that of the uterus, has a low value for the catalytic respiratory systems studied.

Again, the rate of oxygen consumption of certain of the tissue suspensions in the absence of added cytochrome *c* but in the presence of *p*-phenylenediamine (*v* (observed) in table 2) is an appreciable fraction of the full cytochrome oxidase activity. Where this uptake was too small to be measured accurately, the fact is recorded by the designation (>0).

[6] In the preparation of cytochrome oxidase according to Haas (*21*), a certain amount of autolysis of the tissue far from being harmful has indeed been found to be helpful.

TABLE 2.—*Response of suspensions of human normal and neoplastic tissue to added cytochrome c, in the presence of* p*-phenylenediamine (0.2 M)* [1]

Tissue	Hospital No.[2]	V' maximum [3] (Q_c)	V' (observed)[4] (cubic millimeters O_2 per milligram per hour)	Response observed [5]	Cytochrome c	Calculated cytochrome c [6] (mols per liter x 10^6)	v (calculated)[7] (cubic millimeters O_2 per milligram per hour)	Response calculated [8]	Copper
				Percent	*Milligrams percent*			*Percent*	*Milligrams, percent*
Normal tissues: [9]									
Heart muscle		8.2±1.0	4.0±0.4	105	10.2±1.5	6.0	4.0	100	0.3±0.1
Skeletal muscle		2.4± .2	.7± .2	243	2.4± .4	1.6	.5	375	.2± .1
Diaphragm muscle		2.9± .2	.9± .2	222	2.8± .3	1.8	.7	333	.....
Liver		2.8± .3	.8± .1	250	2.3± .4	1.5	.6	400	.8± .1
Kidney		3.6± .4	1.0± .2	260	2.4± .5	1.6	.8	375	.5± .1
Brain		3.9± .2	.9± .1	333	2.3± .4	1.5	.8	400	.3± .1
Thyroid		.6± .1	.1	500	1.0± .2	.8	.08	750	.2± .1
Spleen		.4± .1	(>0)	.....	.8± .1	.6	.03	1,000	.2± .1
Pancreas		.5± .1	.1	.....	.8± .1	.6	.05	1,000	.2± .1
Adrenal		.7± .2	.....	.....	.8± .1	.6	.06	1,000	.3±0
Intestine		.5± .1	.1	.....	.9± .2	.7	.06	857	.2± .1
Stomach		.5± .1	.1	.....	.9± .2	.7	.06	857	.2± .1
Uterus		4	(>0)	.....	.7	.5	.03	1,200	.1
Bladder		.4± .2	(>0)	.....	.7± .2	.5	.03	1,200	.04± 02
Prostate		.2± .1	(>0)	.....	.8± 2	.6	.02	1,000	.2± .1
Lung		.4± .1	(>0)	.....	.7± .1	.5	.03	1,200	.2±0
Neoplastic tissues: [10]									
Thyroid adenoma (fetal) [11]	C-370323	1.42	.22	550	1.6	1.0	.20	600	.4
Thyroid adenomas [12]	M-R3868*	.98	.12	716	1.2	.8	.11	750	.....
Nodular goiter [13]	M-Q8264	.40	(>0)	.....	.9	.6	.04	1,000	.2
Colloid goiter [14]	M-Q8214	.48	(>0)	.....	.9	.6	.05	1,000	.3
Thyroid adenoma, nodular goiter [15]	M-R3296*	.38	(>0)	.....	.9	.6	.04	1,000	.....
Dermatofibrosarcoma protuberans	M-R3411*	.68	(>0)	750	.6	.4	.04	1,500	.....
Hypertrophied prostate	M-Q9091	.42	(>0)	.....	1.2	.8	.03	750	.4
Do	M-R3382	.40	(>0)	.....	.9	.6	.03	1,000	.....
Do	M-Q9090	.40	(>0)	.....	1.3	.8	.04	750	.....
Do	M-Q9114	.42	(>0)	.....	1.4	.9	.05	644	.4
Early prostatic adenocarcinoma	C-374195*	.32	(>0)	.....	.8	.5	.03	1,200	.....
Ovarian tumor granulosa [16]	M-Q9247	.90	.12	650	1.3	.8	.11	750	.2
Uterine fibromyoma	M-Q8380	.34	(>0)	.....	.8	.5	.02	1,200	.2
Do	M-R3307*	.48	.....	.....	.7	.4	.02	1,500	.....
Epiphyseal giant-cell tumor	M-Q8652	.68	.12	466	1.6	1.0	.10	600	.3
Giant-cell tumor possibly malignant	M-Q8905	.54	.12	350	1.3	.8	.07	750	.2
Chondrosarcoma, low grade	C-319745	.68	.12	466	1.0	.6	.07	1,000	.2
Mixed parotid tumor	M-Q8082	.70	.12	483	1.4	.8	.08	750	.3
Synovioma	M-Q8473	.54	(>0)	575	.8	.5	.04	1,200	.3
Unclassified spindle-cell sarcoma	M-R3578*	.48	(>0)	.....	.7	.4	.02	1,500	.....
Unclassified connective-tissue tumor [17]	M-R3622*	.42	(>0)	.....	.6	.4	.02	1,500	.....
Recurrent melanoma (pigmented) [18]	C-350370*	.32	[19] .48	.....	.8	.5	.02	1,200	.....
Melanoma (pigmented) [20]	M-Q8524	.30	[21] 1.20	.....	.7	.4	.02	1,500	.4
Melanoma (nonpigmented)	M-Q9042	.35	(>0)	.....	.7	.4	.02	1,500	.5
Desmoid tumor	M-Q9492	.56	(>0)	.....	.9	.6	.04	1,000	.2
Muco-epidermoid carcinoma	M-R3285*	.34	(>0)	.....	.4	.3	.02	2,000	.....
Epidermoid carcinoma	M-R3360*	.42	(>0)	.....	.3	.2	.01	3,000	.....
Do	M-R3677*	.44	(>0)	.....	.4	.3	.02	2,000	.....
Malignant meningioma	C-327819	.34	(>0)	.....	.3	.2	.01	3,000	.1
Meningioma	C-370369	.32	(>0)	.....	.4	.2	.01	3,000	.1
Bronchogenic carcinoma [22]	Morr.–A-3783	.42	(>0)	.....	.3	.2	.01	3,000	.2
Lymphosarcoma [22]	M-Q9168	.22	(>0)	.....	.3	.2	.01	3,000	.2

See footnotes at end of table.

TABLE 2.—*Response of suspensions of human normal and neoplastic tissue to added cytochrome c, in the presence of* p-*phenylenediamine (0.2 M)* [1]—Continued

Tissue	Hospital No.[2]	V maximum[3] (Q_c)	v (observed)[4] (cubic millimeters O_2 per milligram per hour)	Response observed[5]	Cytochrome c	Calculated cytochrome c[6] (mols per liter x 10^6)	v (calculated)[7] (cubic millimeters O_2 per milligram per hour)	Response calculated[8]	Copper
				Percent	*Milligrams percent*			*Percent*	*Milligrams, percent*
Neoplastic tissues: [10]—Continued[9]									
Early gastric adenocarcinoma	C-369537	0.22	(>0)	.....	0.9	0.5	0.01	1,200	0.2
Gastric adenocarcinoma	M-Q8562	.38	(>0)	.....	.6	.3	.01	2,000	.4
Mucosal gastric carcinoma	C-336902	.28	(>0)	.....	.4	.2	.01	3,000	.3
Gastric adenocarcinoma	M-Q8677	.28	(>0)	.....	.4	.2	.01	3,000	.3
Do	C-371203	.34	(>0)	.....	.4	.2	.01	3,000	.3
Colon adenocarcinoma	M-Q8764	.50	(>0)	.....	.5	.3	.01	2,000	.2
Splenic-flexure adenocarcinoma	C-370059	.48	(>0)	.....	.5	.3	.02	2,000	.3
Cecum adenocarcinoma	C-379946*	.40	(>0)	.....	.4	.2	.01	3,000	.....
Malignant adenoma of sigmoid	M-R3489*	.50	(>0)	.....	.4	.2	.01	3,000	.....
Colon adenocarcinoma	M-R3666*	.48	(>0)	.....	.4	.2	.01	3,000	.....
Sigmoid adenocarcinoma	C-76414	.38	.....	.....	.4	.2	.01	3,000	.....
Rectal adenoma	C-73770	.82	.12	583	.9	.6	.08	1,000	.5
Rectal adenocarcinoma	M-Q8212	.48	(>0)	.....	.7	.4	.03	1,500	.2
Do	C-365663	.36	(>0)	.....	.7	.4	.02	1,500	.2
Do	C-370367	.50	(>0)	.....	.5	.3	.02	2,000	.2
Do	M-Q8729	.44	(>0)	.....	.4	.2	.01	3,000	.3
Do	M-Q9491	.48	(>0)	.....	.4	.3	.02	2,000	.3
Mammary carcinoma	M-Q8168	.48	(>0)	.....	.4	.2	.02	3,000	.2
Do	M-Q8479	.24	(>0)	.....	.2	.1	.005	6,000	.2
Do	M-Q9240	.32	(>0)	.....	.3	.2	.01	3,000	.2
Do	M-Q9522	.36	(>0)	.....	.3	.2	.01	3,000	.2
Do	M-R3297*	.42	(>0)	.....	.4	.2	.01	3,000	.....
Medullary mammary carcinoma	M-R3341*	.28	(>0)	.....	.2	.1	.005	6,000	.....
Infiltrating duct carcinoma	M-R3345*	.28	(>0)	.....	.3	.2	.01	3,000	.....

[1] All data given on basis of wet weight of tissue.

[2] Prefixes M, C, Morr.— refer, respectively, to Memorial, New York, and Morrisania Hospitals.

[3] V maximum (V max.) = Q_c (activity of cytochrome oxidase) = Cubic millimeters O_2 per milligram per hour. Cytochrome c present = 1×10^{-4} M.

[4] v observed (v obs.) = oxygen uptake by tissue suspensions in the presence of p-phenylenediamine and in the absence of added cytochrome c in terms of cubic millimeters per milligram per hour. Values within experimental error are given as (>0).

[5] Response observed $= \frac{V \text{ maximum} - v \text{ (observed)}}{v \text{ (observed)}} \times 100.$

[6] Calculated from observed values.

[7] v (calculated) is obtained from the Michaelis-Menten equation, wherein $Km = 6.0\times10^{-6}$. (See text.)

[8] Response calculated $= \frac{V \text{ maximum} - v\text{(calculated)}}{v \text{ (calculated)}} \times 100 = \frac{Km}{\text{Cytochrome } c} \times 100.$

[9] Averaged values of each of the tissues of the five normal human subjects. (See text.) Tissues from stomach and intestine were cross sections.

[10] Unless otherwise indicated, all tissues studied were obtained within 2 hours after operation. Tissues indicated by asterisk following hospital number were studied fresh; all others were first frozen in dry ice.

[11] 67 percent epithelial cells.

[12] 41 percent epithelial cells.

[13] 12 percent epithelial cells.

[14] 22 percent epithelial cells.

[15] 15 percent epithelial cells.

[16] Considerable luteinization.

[17] Entire kidney replaced by "locally malignant" tumor.

[18] 2.5 percent pigment.

[19] Oxygen consumption in the presence of excess cytochrome c was 0.72 mm.^{3} per hour per milligram of wet tissue; with buffer instead of cytochrome c, it was 0.48, and with 0.01 M KCN instead of either water or cytochrome c, it was 0.40.

[20] 15 percent pigment.

[21] Oxygen consumption in the presence of excess cytochrome c was 1.50 mm.^{3} per hour per milligram of wet tissue; with water or 0.01 M KCN in place of the cytochrome c, it was 1.20.

[22] Material obtained 20 hours after death.

The only data in the literature with which our values may be compared are those by Rosenthal and Drabkin (*10*) on the concentration of cytochrome *c* in the human lung and the kidney cortex. The values for lung (table 2) agree well with those given by these authors, but our values for the kidney are considerably higher than theirs.[7] It may be that this disparity is owing to the fact that we investigated the whole kidney whereas Rosenthal and Drabkin used the cortex only, an explanation which is strengthened by the statement of these authors that the kidney medulla contains more cytochrome *c* than does the cortex (*10*).

Of all the normal tissues studied, liver has by far the greatest concentration of copper (table 2). There does not appear to be any definite correlation of copper content with cytochrome oxidase activity, for the heart muscle, which is the most active tissue, has less copper than the liver. It may be that the liver, in contrast with the heart, may act as a storage center of copper, but there is no evidence to support this concept. On the other hand, it appears to be true that tissues low in cytochrome oxidase activity are on the whole relatively low in copper. Our values for the copper content of the liver and spleen are in substantial agreement with those previously reported (*29*, *30*).

With few exceptions, the values for the activity of cytochrome oxidase and the concentration of cytochrome *c* in the neoplastic human tissues are generally of a low order of magnitude as compared with normal human tissues, such as heart muscle, brain, liver, and kidney (table 2). One of the specimens of thyroid adenoma gave relatively high values for these components. On the other hand, the order of magnitude of these components in the majority of the neoplastic tissues studied is similar to that observed for such a group of normal tissues as bladder, uterus, spleen, and lung.

[7] Since our values are given in terms of wet weight and those of Rosenthal and Drabkin in terms of dry weight, a factor of 4–5 has been used to divide the values they give so as to make the data comparable.

Study of the oxygen uptake of the suspensions in the absence of cytochrome *c* (*v* (observed) in table 2) revealed that little of the total cytochrome oxidase activity of the tissues could be accounted for by the cytochrome oxidase-cytochrome *c* system inherent in the tissues. Where the values were too low to measure the designation (>0) was used.

The copper content of the neoplastic tissues did not appear to vary much from tumor to tumor, the order of magnitude for all these tissues appearing to be about the same.

The only data in the literature with which certain of our results may be compared are those on the concentration of cytochrome *c* in adenocarcinomas of the gastrointestinal tract, for which the average value of 3 mg. per 100 gm. of dry, or roughly 0.6 mg. per 100 gm. of wet weight of tissue, has been reported (*10*).[8] The values given in table 2 for similar tissues are in substantial agreement with this value.

ENZYMATIC ACTIVITY AND THE PROPORTION OF EPITHELIAL CELLS

The tumors studied were heterogeneous, being composed of mixtures in various proportions of cellular and noncellular elements, viz, cancer cells, connective-tissue cells, fibers, etc. Attempts have been made in various ways to correlate the chemical activity of cancer tissues with the cellularity, either by visual estimation under the microscope of the number of such cells or by analysis of the nucleoprotein phosphorus. (See (*10*) for earlier literature.) The employment of what is referred to as nucleoprotein phosphorus for an index of cellularity may be of rough use, but it rests upon somewhat uncertain foundations. The reasons for this lie in (1) the fact that nonlipid phosphorus need not necessarily be nucleoprotein phosphorus, and (2) the eventuality that even if all the phosphorus so measured belonged to nucleoproteins, the value obtained would be the amount of this element in a mixture

[8] See footnote 7.

of cells of varying nucleoprotein concentration, i. e., a tumor composed of a few cells of high nucleic acid content might give the same phosphorus value as a tumor composed of many cells of low nucleic acid content. There is at present no evidence that all cancer cells have the same proportion of nucleic acid, or even that any one kind of cancer cell has the same proportion of this acid at all stages.

What promises to be the most useful and most accurate procedure of determining the relative proportion of the various components present in a sample of tissue is that of Chalkley (*24*). This method depends upon the repeated estimation, at various settings of the microscope, of the proportion of the various tissue components. The technique is free from subjective error, and with a sufficient number of readings it is capable of attaining a high degree of accuracy in the most heterogeneous types of tissue.

The application of Chalkley's method is limited in the present study to the few neoplastic tissues which possess a relatively high enzymatic activity, that is, the thyroid tissues and the pigmented melanomas. The maximum cytochrome oxidase activity exhibited by all the neoplastic tissues studied, with the exception of certain of the thyroid adenomas, was so low, the differences between them so small, and the experimental error consequently so high relatively, that it was considered inadvisable to attempt any correlation of such activity with the relative proportion of epithelial cells. In the thyroid tissues, however, there were several samples with widely varying cytochrome oxidase activity, and it was considered feasible to attempt such a correlation. The data in table 2 reveal a satisfactory correlation. If it is assumed that each epithelial cell in the thyroid tissues possesses the same activity regardless of the source, then by using tissue C–370323 with 67 percent epithelial cells and 1.42 cytochrome oxidase activity as a base line, the activity of the other thyroid tissues may be calculated by simple proportion. The results are as follows: Tissue M–R3868 with 41 percent epithelial cells should give a cytochrome oxidase activity of 0.87, that found was 0.98; M–Q8264 with 12 percent epithelial cells should give an activity of 0.25, that found was 0.40; M–Q8214 with 22 percent epithelial cells should give an activity of 0.46, that found was 0.48; and M–R3296 with 15 percent epithelial cells should give an activity of 0.32, while that found was 0.38.

By the same reasoning, the cytochrome *c* content of M–R3868 should be 1.0, that observed was 1.2; and of M–Q8264 it should be 0.3, that found was 0.9. Likewise the copper in M–Q8264 should be 0.08, that found was 0.18; and in M–Q8214 it should be 0.14, that observed was 0.25. The data for these components are not extensive, but there appears to be little correlation of the cell count with the copper content. In part, the discrepancies observed in certain instances between the calculated and the found values may be due to the ignoring of the contribution by other cellular components of the tissues. In this connection it is interesting that the largest discrepancies of the enzyme and other components studied were in the tissue with the smallest proportion of epithelial cells, M–Q8264.

MELANOTIC MELANOMAS

The oxygen consumption of the suspensions of the highly pigmented melanoma M–Q8524 in the presence of excess cytochrome *c* and *p*-phenylenediamine was unusually high, 1.50 mm.^{3} per milligram wet weight per hour. When the usual control of the suspension in the presence of potassium cyanide was performed, the oxygen consumption value was 1.20 mm.^{3} per milligram per hour. When buffer was used in place of potassium cyanide, the figure was practically unchanged. There exists in this tissue, therefore, a highly active cyanide-insensitive system which oxidizes *p*-phenylenediamine.

Further study of this tumor appeared to be desirable, and the possible presence of tyrosinase and of dopa oxidase

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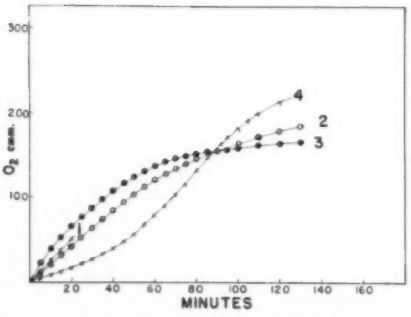

FIGURE 1.—Oxygen consumption of suspensions of a human melanotic melanoma M-Q8524 (table 2). All mixtures contained 1 cc. of tissue suspension equivalent to 100 mg. wet weight of tissue.

Curve 1, 1 cc. of suspension plus 1 cc. phosphate buffer at pH 7.2 plus 1 cc. of cytochrome *c* (1×10^{-4} M). 0.3 cc. *p*-phenylenediamine (0.2 M) in the side arm of the flask;

Curve 2, 1 cc. of suspension plus 1 cc. phosphate buffer at pH 7.2 plus either 1 cc. of the buffer or 1 cc. of 0.01 M potassium cyanide. 0.3 cc. *p*-phenylenediamine (0.2 M) in the side arm of the flask;

Curve 3, 1 cc. of suspension plus 2 cc. phosphate buffer at pH 6.5, 0.5 cc. dihydroxyphenlyalanine (0.01 M) in the side arm;

Curve 4, 1 cc. of suspension plus 2 cc. phosphate buffer at pH 6.5. 0.5 cc. tyrosine (0.01 M) in the side arm.

NOTE.—Controls of the suspension alone or with added tyrosine or dihydroxyphenylalanine in the presence of 1 cc. 0.01 M potassium cyanide yielded negligible consumption of oxygen.

was investigated. One cubic centimeter of a suspension of the tissue (equivalent to 100 mg. of wet tissue) was mixed with 2 cc. of phosphate buffer at pH 6.5 in a Warburg flask and connected with a single manometer. After equilibrium at 38° C. was achieved, 0.5 cc. of an aqueous solution of either tyrosine or dihydroxyphenylalanine (dopa) was added from the side arm of the flask. Readings were then made of the oxygen consumption every 5 minutes for a period of a little over 2 hours. The concentration of the substrates was 0.01 M. The curves describing the uptake of oxygen in these mixtures are given in figure 1.

The curves for the uptake of the tissue suspension mixtures with either tyrosine (curve 4) or dihydroxyphenylalanine (curve 3) reveal that on the basis of a consumption of five atoms of oxygen for the former substrate and of four atoms for the latter, the reactions are nearly complete within the period of time studied. Control runs made of the suspension in water alone, or with tyrosine or dihydroxyphenylalanine in the presence of 1 cc. of 0.01 M potassium cyanide, revealed negligible uptakes of oxygen. Tyrosinase and dopa oxidase are thus cyanide-sensitive systems.

In contrast with tyrosinase and dopa oxidase, the system or systems which oxidize *p*-phenylenediamine in this tumor are practically insensitive to cyanide. The concentration of this substrate in mixtures of the tissue suspension (100 mg.) and phosphate buffer at pH 7.2 was 0.2 M; 0.3 cc. of the substrate solution was added from the side arm (curve 2). Curve 2 shows the oxygen consumption whether 1 cc. of cyanide was present or absent.

The presence of tyrosinase and of dopa oxidase in mouse melanotic melanomas was unequivocally shown by Hogeboom and Adams (*31*). The presence of cyanide-insensitive systems which oxidize *p*-phenylenediamine in mouse melanotic melanomas was first shown by Greenstein and Algire (*32*); in the course of this study it was observed that the amelanotic melanomas of the mouse are completely lacking in any of the systems which oxidize tyrosine, dihydroxyphenylalanine, or *p*-phenylenediamine. Study of the nonpigmented melanoma M-Q9042 (table 2) revealed that addition of cyanide to the suspension of the tissue with *p*-phenylenediamine caused the oxygen consumption of the mixture to become negligible. The presence of the systems which oxidize these substrates is connected with the formation of pigment and not with the fact that they may be observed in a melanoma.[9]

[9] In the course of the interminable controversies over the dopa reaction of Bloch (*33*), it was pointed out that *p*-dimethylaminophenylenediamine was oxidized by pigmented tissues (*34*).

The activity of the highly pigmented tumor M-Q8524 toward all the substrates was far greater than that observed for the melanotic mouse melanomas (*31*, *32*). Another less highly pigmented human melanoma, C-350370 (table 2), showed considerably lower activity toward the substrates; although the oxidation of *p*-phenylenediamine by suspensions of this tumor was vigorous, addition of cyanide caused the rate of uptake to drop from 0.48 to 0.40 mm.^{3} oxygen per milligram per hour. A part of the *p*-phenylenediamine-oxidizing system in this tumor was thus sensitive to cyanide, an observation similar to that made on the pigmented mouse melanomas (*32*). The nature of the cyanide-insensitive systems in the melanotic melanomas, whether of the mouse or of human beings, is not known at the present time. Observations of dilute reduced suspensions of the tumors with a hand spectroscope failed to reveal any trace of the absorption bands characteristic of cytochrome *b*.

The proportion of melanin in melanomas M-Q8524 and C-350370, estimated by the method of Chalkley, was 15 and 2.5 percent, respectively, (by area). It may be presumed that the relative amount of melanin present in these two tissues is a reflection of the respective activity in the tissues of the melanin-forming enzymes. The ratio of melanin in the former to that of the latter is 6:1. The ratio of the activity of the cyanide-insensitive systems which oxidize *p*-phenylenediamine is, for the same tissues, 3:1. The corresponding ratio of tyrosinase is 4:1, and of dopa oxidase 5:1. The correlation of the melanin ratio with those of the tyrosinase and of the dopa oxidase is fairly satisfactory. The ratio of the melanin in the two tissues is not so satisfactorily correlated with the ratio of the *p*-phenylenediamine-oxidizing systems. The direction is the same as that of the other systems, but the order of magnitude is smaller. Whether a better correlation should be expected is perhaps doubtful since the *p*-phenylenediamine-oxidizing system may only be a variable adjunct to the dopa oxidase and the tyrosinase systems which actually produce the melanin. The cytochrome oxidase activity is apparently independent of the presence of melanin or of the melanin-forming enzymes (table 2).

RESPONSE OF TISSUE SUSPENSIONS TO ADDED CYTOCHROME *c* IN THE PRESENCE OF *p*-PHENYLENEDIAMINE

The oxygen-consumption data (given in parentheses in table 2), relating to the uptake by the mixture of tissue suspensions and *p*-phenylenediamine in the absence of added cytochrome *c*, are interesting inasmuch as they reveal the over-all activity of the cytochrome oxidase-cytochrome *c* system in these suspensions. That cytochrome *c* is never present in excess in any of the tissues studied is shown by the fact that when an excess of this component is added, the oxygen consumption is increased. The limiting factor in the respiration of the suspensions in the presence of excess cytochrome oxidase is thus the concentration of cytochrome *c*. This statement is supported by the consideration that the cytochrome oxidase-cytochrome *c* system is composed of an enzyme-substrate complex subject to kinetic interpretation.

In the presence of *p*-phenylenediamine, the addition of increasing amounts of cytochrome *c* to a fixed amount of cytochrome oxidase produces an increasing velocity of oxygen consumption up to a point beyond which further additions are without effect. The maximum velocity reached is directly proportional to the activity of different preparations of cytochrome oxidase or to the actual amount used of the same preparation of oxidase. Stotz, Altschul, and Hogness (*35*) showed that the enzyme-substrate relations under these conditions can be represented by the Michaelis-Menten equation as follows:

$$v=\frac{V \text{ maximum}\times S}{Km+S} \qquad (4)$$

where V maximum is the maximum velocity elicited by the oxidase preparation in the presence of excess cytochrome c, S is the cytochrome c concentration in mols per liter, v is the velocity of oxidation for various concentrations of cytochrome c, and Km is the equilibrium constant of the reaction. The authors (*35*), using the Lineweaver and Burk analysis (*36*) of these relations, calculated a value for Km of 5.8×10^{-6} mols. We have repeated these studies on our preparations of cytochrome oxidase and cytochrome c and have confirmed the work of Stotz, Altschul, and Hogness. Our calculated value for Km is 6.0×10^{-6} mols.

Since the Michaelis-Menten relationship holds in the case of the tissue suspensions, it should be possible to predict the oxygen uptake of such suspensions in the presence of *p*-phenylenediamine (values in table 2) from the values of the measured cytochrome oxidase activity (V maximum), measured cytochrome c content, and Km. The substitution of these values in equation 4 should yield the observed respiration of the tissue suspensions. That this relation comes close to representing the conditions within the suspensions is revealed by the data in table 2.

The values of v (calculated) in table 2, calculated from the Michaelis-Menten equation, are of the same order as those of v (observed), referring to the oxygen consumption of the suspension only in the presence of *p*-phenylenediamine. The rate can, therefore, be closely accounted for in terms of the activity of cytochrome oxidase and the concentration of cytochrome c, in the tissue suspensions. The concentration of cytochrome c, in mols per liter, was calculated for each tissue from the values given in the table, on the basis that all the wet tissue was water; actually, of course, if the solids were subtracted from the tissue weight (or volume), the concentration of cytochrome c would be expressed in higher values, thereby leading to higher v (calculated) values and still better agreement with the values of v (observed). These corrections were not made since the proportions of solids in the various tissues were not estimated. The molecular weight of cytochrome c was taken as 16,000.[10]

The observed response of each of the tissues is given in table 2 in terms of the following equation:

$$\text{Percent response observed} = \frac{V \text{ maximum} - v \text{ (observed)}}{v \text{ (observed)}} \times 100 \quad (5)$$

By "response" is meant the degree to which the particular tissue suspension responds by increased oxygen consumption when excess cytochrome c is added. Since it is clear that in all the tissues studied, the oxygen consumption is limited by the cytochrome c concentration, such response of the tissue will be inversely proportional to the cytochrome c concentration. The calculated percentage response is thus given by the equation:

$$\text{Percent response (calculated)} = \frac{V \text{ maximum} - v \text{ (calculated)}}{v \text{ (calculated)}} \times 100 = \frac{Km}{S} \times 100 \quad (6)$$

where S is the cytochrome c concentration in mols per liter (table 2). The values of v (calculated) and of the percent response (calculated) are uniformly lower than the corresponding observed values but are sufficiently close to justify the methods of calculation and the inferences upon which they are based. As stated previously the small differences between observed and calculated values may be owing to the somewhat arbitrary procedure in calculating the cytochrome c concentration on the basis of mols per liter while neglecting the proportion of solids.

[10] The validity of the Michaelis-Menten equation (table 2) indicates that cytochrome c can in effect be determined in tissues in two ways: (1) By direct analysis of the tissue, i. e., enzymatically (Stotz's method) or spectroscopically; or (2) by the use of the Michaelis-Menten equation, i. e., by substituting therein the measured value of cytochrome oxidase (V maximum), the measured value of v, and the value of Km, and finally solving for S, the concentration of cytochrome c.

RESPONSE TO ADDED CYTOCHROME *C* IN RAT TISSUES

In order to see whether the relations shown to hold in the case of human tissues also hold for the tissues of other species, the data in table 1 have been similarly calculated and are given, together with the data of other investigators, in table 3.

It is clear that the values of v (observed) are very nearly the same as those of v (calculated) in those instances for which the data for cytochrome *c* content and the oxygen consumption of the suspensions are known.

CYTOCHROME OXIDASE IN VARIOUS SPECIES

As a matter of comparative biochemical interest, the cytochrome oxidase of certain tissues of various species were determined and compared. All procedures were the same as those described. The data are given in table 4.

The enzymatic activity appears to be greatest in the tissues of the rat, less in those of the mouse, still less in the tissues of the guinea pig, and least in the tissues of the rabbit and of man. The

TABLE 4.—*Comparative cytochrome oxidase activity (Q_c) of the normal tissues of various species* [1]

Tissue	Cytochrome oxidase activity of—				
	Man	Mouse [2]	Rat [3]	Guinea pig	Rabbit
Liver	2.8	8.0	11.8	4.2	2.9
Spleen	.4	2.0	4.3	.9	.8
Kidney	3.6	11.0	14.0	5.4	4.7
Heart	8.2	18.6	40.2	9.6	9.5
Brain	3.9	9.6	13.0	6.0	4.9
Muscle (skeletal)	2.4	2.8	6.3	1.6	2.0

[1] All data given on tissue-wet-weight basis; average of several determinations. Q_c=cubic millimeters O_2 per milligram per hour.
[2] A and C3H mice. [4] No strain differences were observed.
[3] Buffalo rats.

TABLE 3.—*Response of suspensions of normal and neoplastic tissues of the rat to added excess cytochrome* c

Tissue	Reference	V maximum (Q_c)	v (observed) [1] (cubic millimeters O_2 per milligram per hour)	Response observed [2]	Calculated cytochrome *c* in tissue (mols per liter x 10^{-6})	v (calculated) [3] (cubic millimeters O_2 per milligram per hour)	Response calculated [4]
				Percent			*Percent*
Liver	Table 1	[5] 11.8	7.0	70	5.5	5.6	109
Jensen sarcoma	Table 1	1.6	.22	627	.7	.17	857
Liver	(9)	[5] 30.9			5.0	14.0	120
Hepatoma 31	(9)	6.8			2.0	1.7	300
Heart	(2)	[6] 69.0			33.1	58.4	18
Kidney	(2)	37.2			20.6	28.8	28
Muscle	(2)	15.9			10.0	9.9	61
Brain	(2)	36.0			4.6	15.6	131
Liver	(2)	13.7			4.2	5.6	144
Spleen	(2)	13.1			3.0	4.4	200
Lung	(2)	9.8			1.8	2.3	333
Tumor R256	(2)	23.3			.2	.8	3,000
Spontaneous tumor	(2)	20.8			.1	.3	6,000
Heart	(4, 5)	[7] 1,699			23.2	1,350	26
Kidney	(4, 5)	695			15.4	501	38
Muscle	(4, 5)	271			6.1	136	98
Brain	(4, 5)	476			3.2	165	187
Liver	(4, 5)	479			5.6	231	107
Spleen	(4, 5)	222			2.6	67	231
Lung	(4, 5)	109			1.3	19	461
Flexner-Jobling tumor	(4, 5)	100			.7	10	857
Walker 256 carcinoma	(4, 5)	80			.6	7	1,000
Jensen sarcoma	(4, 5)	148			.7	15	857
Hepatoma	(4, 5)	151			1.2	25	500

[1] Buffer substituted for cytochrome *c*; on basis of wet weight of tissue.
[2] Response observed $=\frac{V \text{ maximum} - v \text{ (observed)}}{v \text{ (observed)}} \times 100$.
[3] v calculated is obtained from the Michaelis-Menten equation, wherein $Km = 6.0 \times 10^{-6}$.
[4] Response calculated $=\frac{V \text{ maximum} - v \text{ (calculated)}}{v \text{ (calculated)}} \times 100$, or $\frac{Km}{\text{Cytochrome } c} \times 100$.
[5] Cytochrome oxidase activity in cubic millimeters O_2 per hour per milligram wet weight of tissue.
[6] Cytochrome oxidase activity in activity units per milligram dry weight. 1 unit causes an increase in oxygen consumption of 10 $mm.^3$ per hour (*2*).
[7] Corrected cytochrome oxidase activity in cubic millimeters O_2 per hour per milligram dry weight of tissue (*5*).

relative order of activity of the tissues in each of the species studied is just about the same. The relatively weak activity of the human tissues is noteworthy.

NORMAL TISSUES OF PATIENTS WITH CANCER

The liver, spleen, and lymph nodes of two patients who died of leukemia were examined after necropsy. The data are given in table 5.

TABLE 5.—*Activity of cytochrome oxidase and the concentrations of cytochrome c and of copper in the tissues of two patients*[1]

Patient	Autopsy No.[2]	Tissue	Cytochrome oxidase (Q_c)	Cytochrome *c*	Copper
				Milligrams pct.	*Milligrams pct.*
P. L.[3]	Q-8931	Spleen	0.30		0.30
		Liver	2.96	2.2	.85
		Lymph nodes	.24		
M. S.[4]	Q-9179	Spleen	.28		.40
		Liver	2.96	2.4	.75
		Lymph nodes	.24		.30

[1] All data given on basis of wet weight of tissue. Q_c=Cubic millimeters O_2 per milligram per hour.
[2] From the Memorial Hospital.
[3] Received extensive X-radiation from 1942 to death (Oct. 29, 1943). Died of leukemia; tissues were obtained 2 hours after death and were frozen in dry ice.
[4] Received X-radiation from Jan. 14, 1942, to Aug. 25, 1943. Died of general lymphatic leukemia; tissues were obtained 2 hours after death and were frozen in dry ice.

Comparison of the data (table 5) with those on the same tissues (table 2) reveals that little if any effect of the leukemic condition was exerted on the activity of cytochrome oxidase and on the concentrations of cytochrome *c* and copper, at least in the tissues studied. Shack (*9*) observed that the cytochrome oxidase activity of the tissues of tumor-bearing animals was unaffected by the presence of the tumor.

DISCUSSION

The data in table 2 illustrate the essential differences between the kinds of tissues. The normal human tissues appear to be divisible into two categories: (1) Heart, skeletal, and diaphragm muscle, liver, kidney, and brain; and (2) the remainder of the normal tissues listed in the table, thyroid, spleen, pancreas, adrenal, intestine, stomach, uterus, bladder, prostate, and lung. The tissues in category 1 have in suspension a high cytochrome oxidase activity, a high cytochrome *c* concentration, and a correspondingly low response value. It will be recalled that the percentage response represents the percent increase in the oxygen consumption in the presence of excess cytochrome *c* and *p*-phenylenediamine over the oxygen consumption of the tissue suspension alone in buffer in the presence of *p*-phenylenediamine. The tissue suspensions in category 2 possess a uniformly low cytochrome oxidase activity and cytochrome *c* content and a correspondingly higher response value. The calculated response range among the tissues of category 1 is 100 to 400, and that among the tissues of category 2 is 750 to 1,200.

The neoplastic-tissue suspensions described in table 2 may also be divided into two categories: category 3, which includes specimens of which the calculated response lies between 600 and 1,200, a range similar to that of category 2 of the normal tissues; and category 4 which includes specimens of which the response range lies between 1,500 and 6,000. Categories 2 of normal tissues and 3 of neoplastic tissues thus overlap. The various categories may be summarized as follows:

Category 1.—Calculated percent response range 100–400. Only normal tissues, i. e., heart, skeletal, and diaphragm muscle, liver, kidney, and brain. Cytochrome *c* content varies from 1.5–6.0 mols per liter.

Categories 2 and 3.—Calculated percent response range 600–1,200. Category 2 is composed of the normal tissues—thyroid, spleen, pancreas, adrenal, intestine, stomach, uterus, bladder, prostate, and lung; and category 3 is composed of the neoplastic tissues—thyroid adenomas, hypertrophied prostates, early prostatic adenocarcinoma, granulosa-cell tumor of the ovary, uterine fibromyomas, giant-cell tumors, chondrosarcoma, mixed parotid tumor, synovioma, desmoid tumor, early gastric adenocarcinoma, and large rectal adenoma. Cytochrome *c* content varies from 0.5 to 1.0 mols per liter.

Category 4.—Calculated percent response range 1,500–6,000. Only neoplastic tissues, including spindle-cell sarcoma, malignant melanomas, epidermoid carcinoma, malignant meningioma, bronchogenic carcinoma, lymphosarcoma, mammary carcinoma, and gas-

tric, colonic, and rectal adenocarcinomas. Cytochrome *c* content varies from 0.1–0.4 mols per liter.

The normal tissues in category 1 are those in which spontaneous neoplasms are less frequently found. The incidence of neoplasia among the tissues of category 2, is relatively high. The neoplasms of category 3 are mostly of a benign variety or else represent an early manifestation of malignancy. The neoplasms in category 4 are all highly malignant. There are some instances of overlapping among the tumors, i. e., one of the samples of melanoma could be placed in category 3, and one of the uterine fibromyomas could be placed in category 4. The dividing line between these categories has of course been arbitrarily drawn, and it would be surprising if there were not a few instances of overlapping. However, it is interesting to observe the classification of the tissues on the basis of the criteria selected. Since the calculated responses of the various tissues are an inverse function of the cytochrome *c* content of the tissues, the categories are based fundamentally on the proportion of this component. Category 1, in which neoplasia occurs infrequently, has a high proportion of cytochrome *c*; category 2, in which neoplasia occurs frequently, and category 3, consisting of neoplasms in a relatively benign state, possess intermediate concentrations of cytochrome *c*; and category 4, consisting entirely of malignant tumors, has the lowest proportion of cytochrome *c*.

In all the tissues of any category, in relation to the content of cytochrome *c*, cytochrome oxidase is present in excess. This excess is relatively small for the tissues of category 1, relatively large for those of category 4, and intermediate for the tissues of categories 2 and 3. In any event, the relationship between cytochrome oxidase and cytochrome *c* may be expressed by the Michaelis-Menten equation as revealed by the data in table 2. As far as the absolute values for cytochrome oxidase activity are concerned, the tissues in category 1 form a single group characterized by relatively high values, while the other tissues, with the possible exception of the thyroid adenomas, form a group characterized in general by values of cytochrome oxidase activity all of the same order of magnitude.

Inspection of the data in table 3 reveals that in analogy with and similar to the case of the human tissues, the rat tissues may be divided into categories: category 1 having the lowest percent calculated response and including only normal tissues, heart and skeletal muscle, liver, kidney, and brain; category 2 containing the normal tissues spleen and lung; and category 3 containing the hepatoma (categories 2 and 3 possess intermediate response values); and finally category 4 having highest response values and consisting only of the neoplasms, tumor R256, the Flexner-Jobling carcinoma, the Walker 256 carcinoma, and the Jensen sarcoma. There is little doubt that the tumors in category 4 are malignant; whether the hepatoma should be classified as benign or malignant has been open to question (*37*).

We have classified the various tissues on the basis of the relation of cytochrome *c* to cytochrome oxidase. It is clear that malignancy is characterized by the widest disparity between the components of the cytochrome oxidase-cytochrome *c* system. In relation to the activity of cytochrome oxidase, malignant tissues have the lowest proportion of cytochrome *c*. It is for this reason that, when to such tissues an excess of cytochrome *c* is added, the response in the form of proportionate increase in oxygen consumption is the greatest. The statement has often been made that malignant tumors are low in cytochrome *c* (*4*, *10*). This statement appears to be generally true. It is of greater interest, however, to point out the relation of such content of cytochrome *c* to the activity of the accompanying oxidase. It may be further pointed out that tumors of a benign type (or less malignant) have no less cytochrome *c* than have a great many normal tissues.

The data in the literature on the respiration of surviving human tissue slices are very meager. Inspection of the data assembled by Burk (*6*) and of the recent observations by Roskelley, Mayer, Horwitt, and Salter (*38*) reveals that the Qo_2 values of normal and neoplastic human tissues may range from 1.0 to 13.3 mm.[3] per hour per milligram dry weight. On a wet-weight basis, this range becomes roughly 0.2 to 2.6. Most of the tissues appear to yield a value near 1.0 on the wet-weight basis, and this value may be adopted as a rough approximation, or better, perhaps, as an order of magnitude. Because of the paucity of the data and the uncertainties inherent in the comparison of absolute respiration data from several independent groups of investigators, the comparison of the data in this paper with the value 1.0 is admittedly hazardous. It is our belief, nevertheless, that this value is at least representative of the tissues studied. With these reservations in mind, we may attempt the following comparison: The maximum cytochrome oxidase activity of the tissues in category 1 (V maximum in table 2) is considerably greater than necessary to account for the respiration on the basis of the value of 1.0. In these tissues, cytochrome *c* is not present in excess, and consequently the maximum cytochrome oxidase activity is not observed. If the assumption is made that the cytochrome oxidase and cytochrome *c* interact in the tissue as they do in suspension, i. e., if we neglect the role of the structure of the cell, the respiration of the tissues in category 1 can be nearly accounted for on the basis of the cytochrome *c* present (v (observed) in table 2). Heart muscle contains a considerable reserve of cytochrome *c* for the value of v (observed) (table 2) is much greater than necessary to account for the value of 1.0 for the respiration. The tissues in category 1, heart, skeletal, and diaphragm muscle, liver, kidney, and brain, apparently possess either enough or more than enough cytochrome *c* and cytochrome oxidase to account for their respiration. These tissues are highly active metabolically.

On the other hand, the maximum activity of cytochrome oxidase in the tissues of categories 2 and 3 (with the exception of two of the thyroid adenomas) and 4 is generally lower than the value of 1.0 for the respiration of the tissues, while the oxygen consumption of the suspensions due only to the actual amount of cytochrome *c* present (v (observed) or v (calculated) in table 2) is only a very small fraction of that necessary to account for the tissue respiration. This observation is particularly true for the malignant tissues of category 4. Somewhat similar observations may be made in the case of the rat tissues (table 3). The maximum cytochrome oxidase activity of rat liver (V maximum) and the consumption of oxygen limited by the cytochrome *c* concentration (v (observed) or v (calculated)) are more than sufficient to account for the respiration of this tissue. As Shack (*9*) pointed out earlier, this tissue possesses an oxidative reserve. On the other hand, in the case of the Jensen rat sarcoma, the maximum cytochrome oxidase activity is barely more than the value of the respiration for this tissue while the oxygen consumption limited in the suspension by the cytochrome *c* concentration (v (observed)) is considerably less than that of the respiration of the tissue slices.

The disparity between the respiration value for the tissues in categories 2, 3, and especially 4 and the cytochrome respiration may be owing to either one of two factors or to both: (1) That it is necessary to take into account the structure and integrity of the cell, i. e., comparisons between whole cells and suspensions are extremely limited if not improper; and (2) that the respiration of the tissues includes contributions by other systems in addition to that of cytochrome. If there is any validity to the second of these factors, it would appear that the cytochrome system plays only a minor role in the respiration of malignant neoplasms. The utilization of oxygen by these tissues

for the burning of substrates must proceed mainly by systems other than cytochrome. Further speculation along these lines would be unprofitable in view of the limited data now available.

Greenstein and Thompson (*16*) assembled data on a number of enzyme systems in a wide variety of normal and neoplastic tissues of mice. These data revealed that the range of activity of each catalytic system was much narrower among the tumors than among the normal tissues. The view was expressed that from the standpoint of enzymatic catalysis "tumors resembled each other more than they resembled normal tissues, or than normal tissues resembled each other." Independently of the etiology of the tumor, or of the tissue from which it arose, or of the strain of animal in which it was found, the enzymatic activities of the tumors appeared to converge to those of a common type, i. e., the diversity of pattern of the normal tissues was absent in the tumors.

The range of cytochrome oxidase activity among the normal human tissues (table 2) was from 8.2 for heart to 0.2 for the prostate, and the corresponding range among the malignant neoplasms from 0.48 to 0.22. If all the neoplastic tissues are considered, the range was 1.42–0.22. In any case, the range among the normal tissues was much greater than among the neoplasms. The malignant neoplasms appeared to have an extremely narrow range of cytochrome oxidase activity, a feature of this class of tissues which is independent of the tissues of origin and which appears to hold for both human and for mouse tissues.

The range of cytochrome *c* concentrations in the normal human tissues was 10.2–0.7 (table 2); among the malignant human neoplasms this range was 0.4–0.2; among all the neoplastic tissues, benign and malignant, the corresponding range was 1.6–0.2. Again, the range of this component was greater among the normal tissues than among the neoplasms.

The copper content of the normal human tissues varied from 0.04 mg. percent for bladder to 0.8 for liver; the range among all the neoplasms was 0.1–0.5 mg. percent. Again the range among the neoplasms was considerably narrower than among the normal tissues.[11]

The tissues, both normal and neoplastic, of the human being possessed lower cytochrome oxidase activity and cytochrome *c* concentration than did the comparable tissues of the other species studied. However, the ratio of the response values of certain normal to certain neoplastic tissues was essentially similar in both rat and human. Thus the ratio of the calculated percent response values of liver to Jensen sarcoma in the rat was roughly 1:8 (table 3); and the corresponding ratio of human liver to the malignant human neoplasms ranged from 1:4 to 1:15 (table 2). The median value for human tissues was thus close to the value given for the rat tissues.

SUMMARY

About 80 normal human tissues of various kinds and about 60 human neoplasms were examined for cytochrome oxidase activity and cytochrome *c* content. In certain of the normal and neoplastic tissues, the copper content was also determined. The cytochrome oxidase activity and the cytochrome *c* content of the tissues appeared to be largely independent of the time of standing before the tissues were tested (maximum period, 4 hours), of freezing in dry ice prior to testing, or of the death of the host by inhalation of carbon monoxide. Control experiments on the normal and neoplastic tissues of the rat were likewise made, yielding results in accord with those on human tissues.

The rate of oxygen consumption by the tissue suspensions in buffer and in the presence of *p*-phenylenediamine was determined. It was shown that

[11] Robertson (*39*) showed that the ascorbic acid content of a variety of animal tumors was much narrower in range than that of a number of normal animal tissues.

this rate could be closely predicted by the Michaelis-Menten equation from the values of the actual cytochrome *c* content of the tissues, the cytochrome oxidase activity (rate of oxygen uptake in the presence of added excess cytochrome *c*), and the Michaelis-Menten constant. The rate of oxygen consumption by the tissue suspensions was limited by the cytochrome *c* concentration; cytochrome oxidase was always present in excess. When excess cytochrome *c* was added to the various tissues, the rate of oxygen consumption increased, and this proportionate increase fell within certain numerical limits for certain kinds of tissues.

Four categories were made based upon this proportionate increase or percent response as follows:

Category 1, in which the percent response ranged from 100 to 400, included only the normal tissues—heart, skeletal, and diaphragm muscle, liver, kidney, and brain.

Categories 2 and 3, in which the percent response ranged from 600 to 1,200, included, respectively, the normal tissues (thyroid, spleen, pancreas, adrenal, intestine, stomach, uterus, bladder, prostate, and lung) and the neoplastic tissues (thyroid adenomas, hypertrophied prostates, early prostatic adenocarcinoma, granulosa-cell tumor of the ovary, uterine fibromyomas, giant-cell tumors, chondrosarcoma, mixed parotid tumor, synovioma, desmoid tumor, early gastric adenocarcinoma, and large rectal adenoma).

Category 4, in which the percent response ranged from 1,500 to 6,000, included only the neoplastic tissues: spindle-cell sarcoma, malignant melanomas, epidermoid carcinoma, malignant meningioma, bronchogenic carcinoma, lymphosarcoma, mammary carcinoma, and gastric, colonic, and rectal adenocarcinomas.

A few of the tumors overlapped these categories; but it may be stated that in general the normal tissues in category 1 were those in which spontaneous neoplasms are less frequently found, the incidence of neoplasia among the tissues of category 2 was relatively high, the neoplasms of category 3 were mostly of a benign variety or else represented an early development of the malignant state, and finally the neoplasms in category 4 were all highly malignant. It is possible, therefore, to describe groups of normal and neoplastic tissues in terms of their cytochrome *c* content. In this regard, an entire series of normal and of neoplastic tissues, 2 and 3, fell in the same category.

It is pointed out that rat tissues may be classed in similar low, medium, and high categories, i. e., heart and skeletal muscle, liver, kidney, and brain in category 1, spleen and lung in category 2, the hepatoma in category 3, and tumor R256, the Flexner-Jobling carcinoma, and the Jensen sarcoma in category 4.

The possible relation of the respiration of the various surviving tissues to the cytochrome respiration of the corresponding suspension was discussed. The assumed respiration of heart, skeletal, and diaphragm muscle, heart, kidney, and brain tissue may be readily accounted for in terms of the cytochrome *c*-cytochrome oxidase system. On the other hand the cytochrome system of all the other tissues, and especially that of the malignant neoplasms, accounted for only a small fraction of the respiration of these tissues in intact cellular form.

The activity of cytochrome oxidase of the normal human tissues in category 1 was of the same high order of magnitude for all the tissues; the activity of this enzyme in the tissues of categories 2, 3 (with the exception of certain of the thyroid adenomas), and 4 was lower, and of very nearly the same order of magnitude throughout. Taken as a whole, the range of activity of cytochrome oxidase and of concentration of cytochrome *c* was much narrower among all the neoplastic tissues than among all the normal tissues.

The cytochrome oxidase activity of the same kinds of normal tissue in various species of animals was determined. Human tissues gave the lowest

values. The ratio of cytochrome *c* in the liver to that of the Jensen sarcoma in the rat was roughly 8:1, and the ratio of this component in the liver to that in the malignant group of human neoplasms was 4–15:1.

The determinations of copper in the normal and neoplastic human tissues revealed little of significance. Liver possessed the highest value of all the tissues studied. The copper content of the neoplasms was of the same order of magnitude for all the tissues; the range of values for this element among the neoplasms was thus considerably narrower than that for the normal tissues. There appeared to be little if any correlation between the copper content of a tissue and the cytochrome oxidase activity of the latter.

The cytochrome systems in the liver, spleen, and nodes of patients dead of cancer had very nearly the same activity as that of comparable tissues in normal individuals.

The proportion of epithelial cells in a number of samples of neoplastic thyroid tissue was determined, and it was shown that a fairly direct correlation exists between the number of such cells and the cytochrome oxidase activity and cytochrome c content of the respective tissues. No such correlations could be observed in the case of the copper content of the tissues.

Studies of pigmented human melanomas revealed the presence of active, cyanide-sensitive systems in these tissues which oxidized tyrosine and dihydroxyphenylalanine to melanin, and of cyanide-insensitive systems which oxidized *p*-phenylenediamine. The activity of these systems in these tissues was roughly reflected in the respective proportion of melanin within the tissues, a nearly direct correlation of such content with dopa oxidase being observed. The nonpigmented melanoma was lacking in these systems. Both pigmented and nonpigmented melanomas possessed nearly the same cytochrome oxidase activity.

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